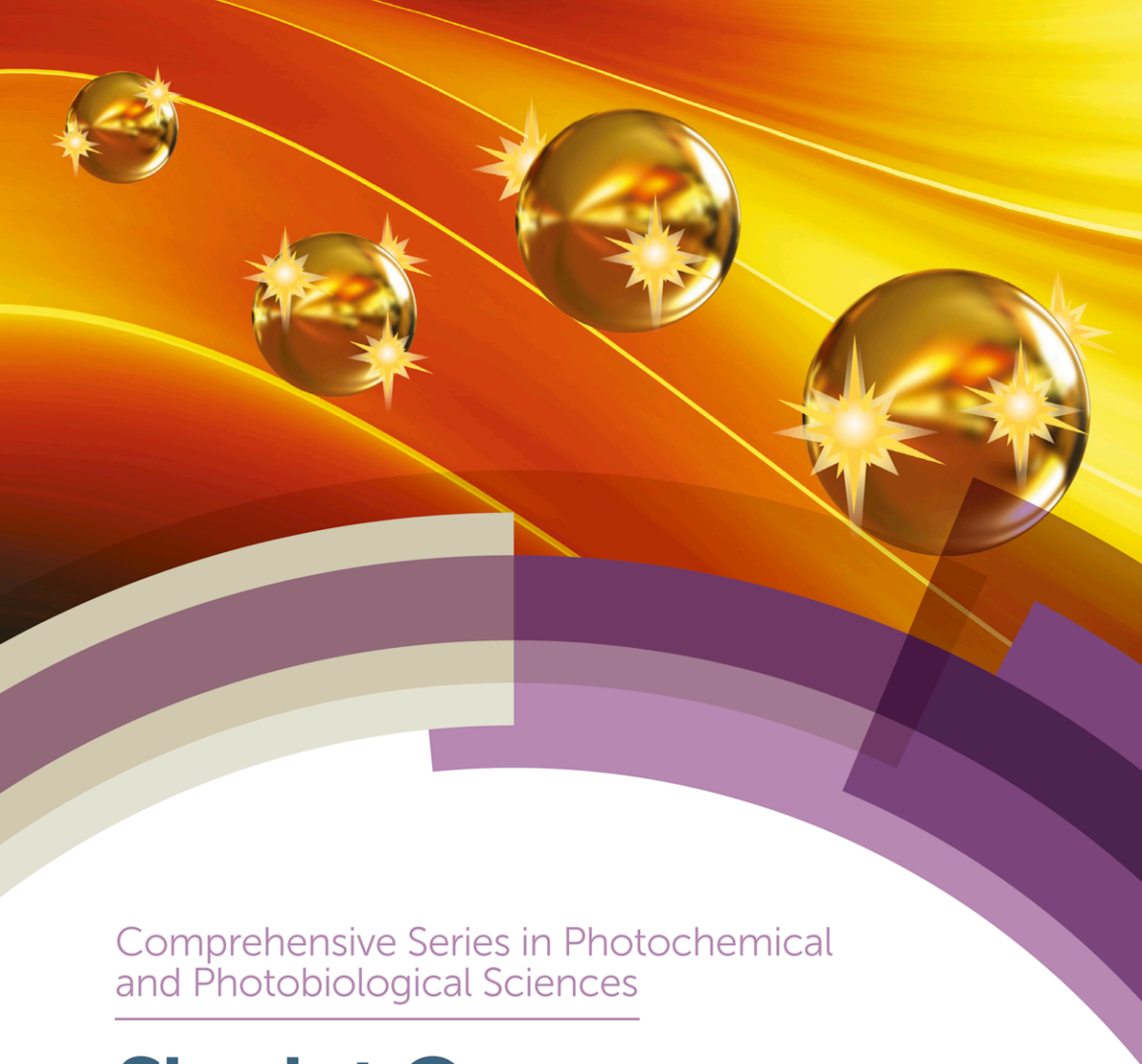




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Singlet Oxygen

Applications in Biosciences and Nanosciences
Volume 1

Edited by Santi Nonell and Cristina Flors



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COMPREHENSIVE SERIES IN PHOTOCHEMISTRY AND
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Singlet Oxygen

Applications in Biosciences and Nanosciences

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To Anna

To Jordi, Elisenda and Miriam

SN

To my parents Rosie and Fernando, and my husband Tom

CF

Preface

Singlet oxygen, the metastable lowest electronically excited form of the dioxygen molecule, has remained a central research subject since its discovery by Kautsky in 1931. Many reasons account for this. First, this small, nonionic, nonradical form of molecular oxygen meets many of the requirements for a formidable reactive intermediate: its small size allows it to diffuse very easily across exceedingly “crowded” systems such as polymers and cellular structures. Secondly, its unique electronic structure and excess energy make it highly reactive against a large variety of electron-rich substrates. Finally, its relatively long lifetime, from seconds in the gas phase to a few microseconds in water, gives it plenty of time to reach to and react with remote targets.

Three decades have elapsed since a book devoted to singlet oxygen was last published,¹ yet the advances and knowledge gained during this period are so vast and so wide reaching that many felt the time had come for an update. With this spirit in mind, we accepted the challenge posed by the Series Editor Giulio Jori to bring together the community of singlet oxygen researchers and convey their hard-won knowledge into a book that should inspire others entering the field. Giulio did not live to see his book published yet its publication is a tribute to his memory.

The book is divided into five major blocks, which can be read independently. Volume 1 begins with Section I, Fundamentals, giving an overview of the basic facts about singlet oxygen and places it in the context of other reactive oxygen species. Section II, Production of Singlet Oxygen, discusses extensively the ways, materials and techniques used to produce singlet oxygen. We have placed special emphasis on production methods that employ light as energy source as these are most relevant for photonic applications. Section III describes the reactivity of singlet oxygen. The basic reactions are underlined, synthetic applications are beautifully exemplified, and the specific details of singlet oxygen reactivity towards materials and biological components are systematically

explored in a series of chapters. To complement this section on reactivity, we encourage our readers to download the review on photo-oxidation of proteins by Michael J. Davies and coworkers.² This article has been made free to access to accompany this book.

Volume 2 begins with Section IV, which describes the known strategies and techniques to detect and monitor singlet oxygen, ranging from purely spectroscopic methods to the use of chemical traps, spin traps, and fluorescent probes, to the issues pertinent to monitoring the dose of singlet oxygen delivered in photodynamic treatments. Finally, Section V covers the current most significant applications of singlet oxygen science. The reader will find these chapters particularly inspiring as they elegantly demonstrate the importance of singlet oxygen for practical applications in the biosciences and the nanosciences.

We have made every effort to unify nomenclature, abbreviations and symbols throughout the book, as well as to minimize overlap and repetitions between the different chapters. As this is a multiauthor book, avoiding such problems has not always been possible. We beg the reader's indulgence for it and hope this does not detract from the overall value of the book.

Finally, a few acknowledgements. First and foremost, a big THANK YOU to Giulio Jori for persuading us to undertake the fantastic task of conveying the state-of-the-art in singlet oxygen research into a single book. The journey has been incredibly rewarding as it has allowed us to interact with top scientific colleagues from around the world. Giulio Jori, together with Silvia Braslavsky and Christopher Foote, have been mentors and friends for many years and, as far as singlet oxygen is concerned, the giants who inspired our work and on whose shoulders we still stand. Next, special thanks also to the authors who have contributed to the book. This is your book. We are indebted to you for your insightful science, your commitment and your patience. We wish to extend our gratitude to you reader. This book was designed for you and with you in mind. It is our deepest hope that it helps you attain a sound understanding of the singlet oxygen science and that it inspires you to push its frontiers further away. Last but not least, we would also like to thank our publisher, the Royal Society of Chemistry, and in particular Janet Freshwater, Vicki Marshall, Antonia Pass and Stefan Turner, for the encouragement, advice, and assistance we have received throughout the process of editing this book.

Santi Nonell and Cristina Flors

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Foreword

It is a pleasure to commend this book *Singlet Oxygen*. The Editors are certainly to be congratulated on persuading so many international experts in this area to contribute chapters on a great variety of topic and of a high scientific standard. I am sure this state-of-the-art volume will become an indispensable reference source for many years to come for those interested in, and researching, singlet oxygen.

In 1957 when I started research on electronic energy transfer in solution as a research student with Professor George Porter, later to become Sir George, then Lord Porter and Nobel Laureate, lasers had not been invented. In order to detect triplet states, the lowest excited state of most molecules, exciting with flashlamps of microsecond duration, oxygen had to be rigorously removed from all solutions, since oxygen was known to efficiently quench electronically excited states. Although Kautsky and de Bruijn¹ had proposed quenching produced singlet oxygen as a reactive intermediate in dye-sensitized photo-oxygenations as early as 1931 it was not until 1964 that this became generally accepted when Foote and Wexler² and Corey and Taylor³ demonstrated that the oxygenation product distribution for several substrates from chemically generated (using $\text{H}_2\text{O}_2/\text{NaOCl}$) and from radio-frequency generated singlet oxygen, was the same as in sensitized photo-oxygenation of these same substrates. In 1968, Foote and Denny⁴ showed that the sensitized photo-oxygenation of 2-methylpent-2-ene is reduced markedly in the presence of beta-carotene and by assuming energy transfer from singlet oxygen to beta-carotene was diffusion controlled with a rate constant of $3 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ they estimated that the lifetime of singlet oxygen was about $10 \mu\text{s}$ in benzene solution. This value was in striking agreement with the value of $12.5 \mu\text{s}$ obtained three years later from time-resolved measurements in my laboratory⁵ using a laser flash photolysis system.

By 1981 James Brummer and I⁶ in a critical comprehensive compilation of the literature were able to report lifetimes in 50 different solvents and second-order rate constants for deactivation and chemical reaction of singlet oxygen with 690 different compounds. The update⁷ in 1995 (20 years ago) reported lifetimes in 145 solvents and solvent mixtures and rate constants for chemical reaction and deactivation of singlet oxygen with 1900 different compounds. This demonstrates the ever increasing interest by scientists in the properties and reactions of singlet oxygen.

As pointed out in the preface it is 30 years since a book was published on singlet oxygen. However, as the many references at the end of the chapters in this book demonstrate research concerning singlet oxygen continues to grow. It is not surprising therefore that the Series Editor Giulio Jori should have encouraged the editors to produce a book on singlet oxygen, a research area in which he made many important contributions. It is a great pity he did not live long enough to see the book in print.

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Section I

Fundamentals

Chapter 1

Overview of Reactive Oxygen Species

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1.1. Molecular Oxygen and Reactive Oxygen Species, an Introduction

The increasing concentration of molecular oxygen (O_2) in the atmosphere roughly 2.5 billion years ago,^{1,2} due to oxygenic photosynthesis by cyanobacteria, allowed for the evolution of aerobic respiration, leading to the development of complex eukaryotic organisms.² For all currently living aerobic species, molecular oxygen is a central molecule in cellular respiration. Certain derivatives of oxygen are, however, highly toxic to cells. In the 1950s, Gerschman *et al.* proposed that oxygen-containing free radicals were responsible for toxic effects in aerobic organisms.^{3,4} Over the years, the terms ROS (reactive oxygen species), ROI (reactive oxygen intermediates) and RNS (reactive nitrogen species) have been coined to define an emerging class of endogenous, highly reactive, oxygen- (and also nitrogen-) bearing molecules. According to some definitions the term ROI describes the chemical species formed upon incomplete reduction of molecular oxygen, namely superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$), while ROS includes both ROI and ozone (O_3) and singlet oxygen ($^1O_2$).⁵ A somewhat more encompassing definition also includes within ROS compounds such as hypochlorous (HOCl), hypobromous (HOBr), and hypoiodous acids (HOI). Incorporation of peroxy ($ROO^{\cdot}$), alkoxy ($RO^{\cdot}$), semiquinone ($SQ^{\cdot-}$) and carboxylate ($CO_3^{\cdot-}$) radicals and organic hydroperoxides (ROOH) is also frequently encountered within the definition of ROS.^{6,7} ROS may also be classified as free radicals and nonradical species.^{7,8} RNS that bear oxygen atoms include nitric oxide radical (NO or $NO^{\cdot}$), nitrogen dioxide radical ($NO_2^{\cdot}$), nitrite (NO_2^-), and peroxynitrite ($ONOO^-$).⁵

Reactive oxygen species, in particular hydroxyl and peroxy radicals, hydrogen peroxide and superoxide radical anion, have long been implicated in oxidative damage inflicted on fatty acids, DNA and proteins as well as other cellular components.⁹ ROS overproduction is associated with numerous disorders.¹⁰ Oxidative stress caused by the imbalance between excessive formation of ROS and limited antioxidant defences is connected to many pathologies including age-related disorders, cancer, cardiovascular, inflammatory, and neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.¹¹⁻¹⁴ According to the long-held "free radical theory of aging"^{15,16} advanced by Denham Harman in 1956, the noxious effects of ROS, generated during cellular respiration at the mitochondrial level are directly involved in aging processes. This hypothesis is, however, currently under revision.⁹ Mounting evidence suggests that ROS actually may have a beneficial physiological role acting as messengers in cellular signaling, a new paradigm in the rich and diverse chemistry of ROS which has attracted increased attention in the last decade (see also Figure 1.1).^{2,8,9,12,17-20} The redox regulation typically involves controlled production of reactive oxygen and nitrogen species. They can, in turn, react with specific functional groups of target proteins (*e.g.* [Fe-S] clusters, cysteines, *etc.*) that lead to covalent protein modifications.²¹ ROS as second messengers are important for the expression of several transcription

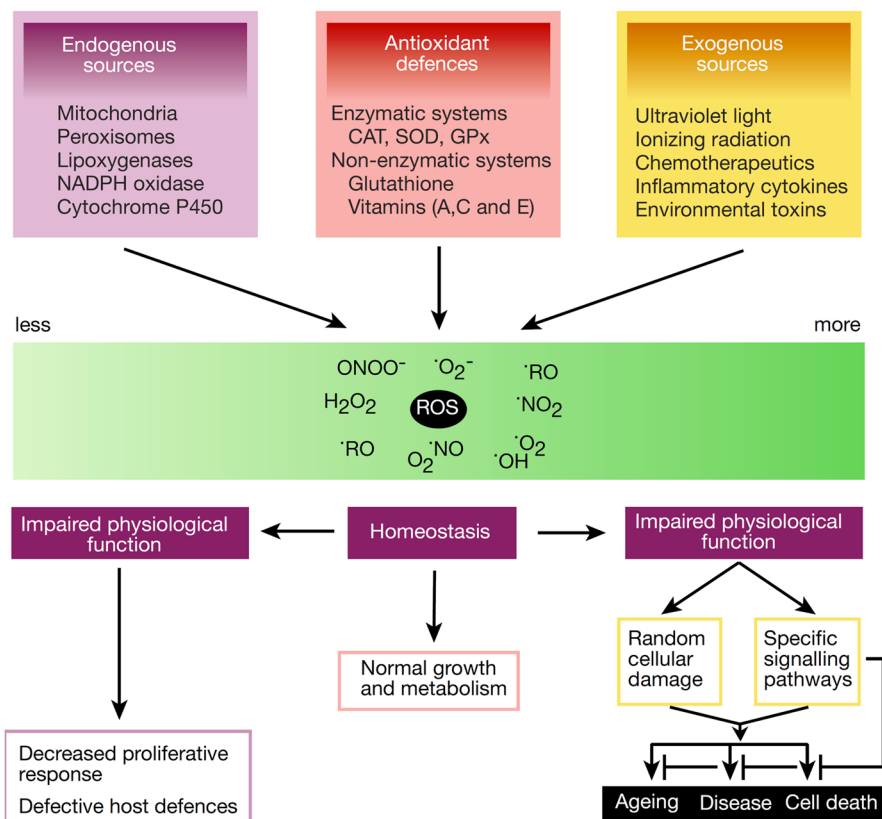


Figure 1.1. Sources of ROS, antioxidant defences, and subsequent biological effects depending on the level of ROS production. Reprinted by permission from Macmillan Publishers Ltd: *Nature*,¹⁰ copyright 2000.

factors and other signal transduction molecules such as heat shock-inducing factor and nuclear factor. They also participate in the regulation of cell adhesion, redox-mediated amplification of immune response and programmed cell death.⁸

Deciphering the highly complex and diverse impact of ROS chemistry in biological environments requires a multidisciplinary approach where chemists may be actively involved providing new tools to generate and detect ROS and their byproducts with spatiotemporal control and precision. Knowledge is, however, required on the chemical properties of these species, their sources both endogenous and exogenous, their typical scavengers and the characteristic lifetimes in order to understand how, when and where they form, how far they travel, what are their targets and how we may control their activity. The following sections summarize current literature on these topics providing a glimpse of what we believe is a most intriguing, challenging and stimulating contemporary problem, that of the biological impact of ROS chemistry.

1.2. Chemical Properties of ROS

In order to understand the chemical and the associated biological impact of ROS, knowledge on their origin, type, and reactivity (specifically, their electronic configuration and redox potential) is required.

1.2.1. Electronic Configuration

Ground-state triplet molecular oxygen is a paramagnetic biradical with two electrons occupying separate π^* orbitals with parallel spins (Figure 1.2). Most nonradical organic molecules are diamagnetic, with pairs of electrons with opposite spins. A spin restriction applies for molecular oxygen to participate in redox reactions with other atoms or molecules as it has to accept, from the reductant, a pair of electrons that have the same spin (*i.e.* nondiamagnetic) so they can fit into the vacant spaces in the π^* orbitals of oxygen. Oxygen is thus unable to efficiently oxidize biomolecules *via e.g.* addition (2-electron process).

The spin restriction results in oxygen preferably accepting one electron at a time during redox reactions. Thus molecular oxygen can react fast with other radicals by single-electron transfer. It may also react with other species bearing unpaired electrons *e.g.* transition metals such as Fe found in [Fe-S] clusters.²² The one-electron reduction of oxygen results in the formation of superoxide radical anion ($\text{O}_2^{\cdot-}$). One-electron reduction of $\text{O}_2^{\cdot-}$ leads to the formation of other ROS such as hydrogen peroxide (H_2O_2) that is a closed-shell molecule (Scheme 1.1, also Figure 1.2). Reduction of hydrogen peroxide in turn yields the hydroxyl radical ($\text{OH}^{\cdot}$) that undergoes reduction to yield water (or hydroxide OH^-).

Ground-state or molecular oxygen O_2 can be, however, converted to more reactive oxygen containing forms. Energy transfer to O_2 leads to the formation of the more reactive molecular oxygen form, singlet oxygen ($^1\text{O}_2$), amply discussed in the following chapters. Singlet oxygen has paired electrons with opposite spins (Figure 1.2). Thus the spin restriction is removed, increasing the oxidizing ability of $^1\text{O}_2$ compared to ground-state O_2 .

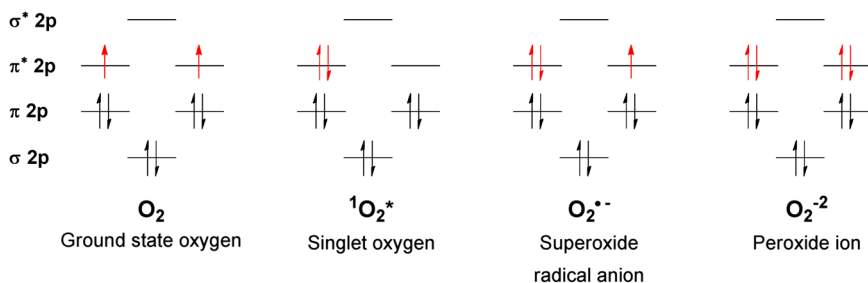
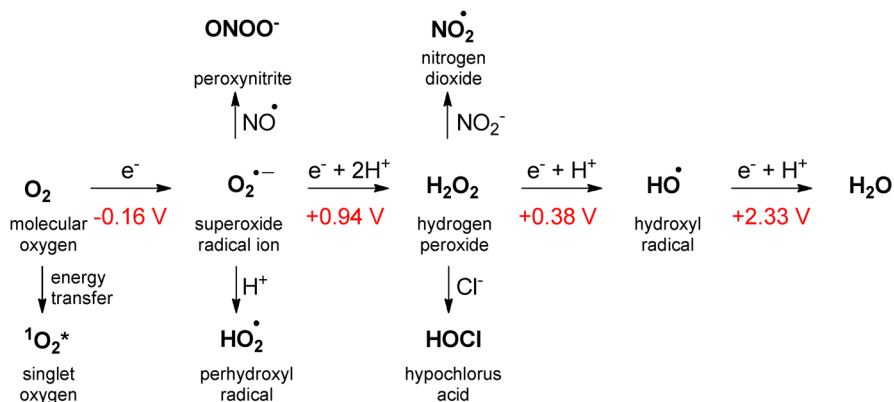


Figure 1.2. Molecular orbital diagrams for ground-state molecular oxygen (O_2), singlet oxygen ($^1\text{O}_2$), and ROS (superoxide radical anion $\text{O}_2^{\cdot-}$ and peroxide ion O_2^{2-} , deprotonated form of hydrogen peroxide H_2O_2).⁹



Scheme 1.1. Formation of ROS through energy- and electron-transfer reactions. The redox states of oxygen with standard reduction potentials are shown. The standard concentration of oxygen was regarded as 1 M, adapted from ref. 22.

1.2.2. Redox Potentials

Knowledge of the thermodynamics of free radical reactions is necessary towards understanding the direction of the electron transfer. Redox potentials of the various ROS intermediates involved in the reduction of molecular oxygen to water are listed in Table 1.1, see also Scheme 1.1. Table 1.1 additionally lists, organized from highly oxidizing to highly reducing, the one-electron redox potentials of various other ROS of biological importance, as well as the one electron redox potential of ROS scavengers, as originally compiled by Buettner.²³

The biradical nature of oxygen restricts it to accepting electrons one at a time during a redox reaction with spin-paired molecules (see above). Molecular oxygen, with a redox potential of -0.16 V (for oxygen concentration of 1 M, pH 7 as the standard state, -0.33 V for 1 bar as the standard state²⁴), is, however, a poor univalent electron acceptor (see Scheme 1.1).²² Consequently, molecular oxygen itself is a poor oxidant and is fairly benign to biomolecules. The unpaired electrons of oxygen may, however, interact with unpaired electrons of transition metals and organic radicals. While one-electron reduction of molecular oxygen to superoxide radical anion is thermodynamically less favorable than its direct two-electron reduction to hydrogen peroxide ($+0.30 \text{ V}$), the simultaneous $2e^-$ requirement of the latter is unfavorable.²⁵ Formation of superoxide radical anion but not hydrogen peroxide is thus characteristic of auto-oxidative processes.

The superoxide radical anion has limited reactivity with electron-rich centers because of its anionic charge.²⁶ Upon protonation of $\text{O}_2^{\bullet -}$ the perhydroxyl radical is obtained ($\text{p}K_a = 4.8$ ²⁷). The new species has an increased reduction potential ($+1.06 \text{ V}$ ²³) and is a better oxidant. The biological relevance of the perhydroxyl radical is, however, believed to be minor given its low concentration at physiological pH. Despite its high reduction potential of $+0.94 \text{ V}$, $\text{O}_2^{\bullet -}$ can oxidize very few biological compounds. One-electron reduction of $\text{O}_2^{\bullet -}$ leads to the formation of hydrogen peroxide.

Table 1.1. One electron redox potential of ROS and ROS scavengers, as originally compiled by Buettner,²³ relative to the standard hydrogen electrode.

Couple	E^0/V
$\text{HO}^\bullet, \text{H}^+/\text{H}_2\text{O}$	2.33
$\text{O}_3^{\bullet-}, 2\text{H}^+/\text{H}_2\text{O} + \text{O}_2$	1.80
$\text{RO}^\bullet, \text{H}^+/\text{ROH}$ (aliphatic alkoxy radical)	1.60
$\text{HOO}^\bullet, \text{H}^+/\text{H}_2\text{O}_2$	1.06
$\text{ROO}^\bullet, \text{H}^+/\text{ROOH}$ (alkylperoxy radical)	1.00
$\text{O}_2^{\bullet-}, 2\text{H}^+/\text{H}_2\text{O}_2$	0.94
$\text{RS}^\bullet/\text{RS}^-$ (cysteine)	0.92
$\text{O}_3/\text{O}_3^{\bullet-}$	0.89
$^1\text{O}_2/\text{O}_2^{\bullet-}$	0.65
Catechol- $\text{O}^\bullet, \text{H}^+/\text{catechol-OH}$	0.53
α -Tocopheroxyl $^\bullet, \text{H}^+/\alpha$ -tocopherol ($\text{TO}^\bullet, \text{H}^+/\text{TOH}$) (vitamin E)	0.50
Trolox C ($\text{TO}^\bullet, \text{H}^+/\text{TOH}$)	0.48
$\text{H}_2\text{O}_2, \text{H}^+/\text{H}_2\text{O}, \text{OH}^\bullet$	0.32
Ascorbate $^\bullet, \text{H}^+/\text{ascorbate monoanion}$ (vitamin C)	0.28
Semiubiquinone, $\text{H}^+/\text{ubiquinol}$ ($\text{CoQ}^{\bullet-}, 2\text{H}^+/\text{CoQH}_2$)	0.20
Ubiquinone, $\text{H}^+/\text{semiubiquinone}$ ($\text{CoQ}/\text{CoQ}^{\bullet-}$)	-0.036
Dehydroascorbic/ascorbate $^{\bullet-}$	-0.17
$\text{O}_2/\text{O}_2^{\bullet-}$	-0.33
Methyl viologen ($\text{MV}^{2+}/\text{MV}^{\bullet+}$)	-0.45
$\text{O}_2, \text{H}^+/\text{HO}_2^\bullet$	-0.46
$\text{RSSR}/\text{RSSR}^{\bullet-}$ (cysteine or glutathione)	-1.50

Although hydrogen peroxide has a positive one electron reduction potential (+0.32 V²³ to +0.38 V^{22,25} based on the source), and an even more favorable two-electron reduction potential (+1.35 V²⁵), it is relatively stable under physiological conditions (slow reaction). In stark contrast, the hydroxyl radical, with a one-electron reduction potential of +2.33 V, is a most powerful oxidant reacting at diffusion control rates with organic matter.²⁸

1.3. Sources of ROS

1.3.1. Endogenous Sources of ROS

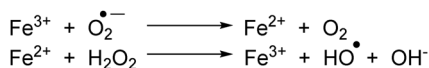
ROS can either be generated exogenously or intracellularly from numerous sources. They are produced in a wide range of biochemical and physiological processes (Figure 1.1). Several different enzymes have been implicated in the generation of ROS.

Cytosolic enzyme systems contributing to the generation of ROS, among others, are the seven isoforms of the expanding family of transmembrane NADPH oxidases (NOXs), a superoxide-generating system.^{29,30} The cytosolic domains of NOX transfer an electron from NADPH to a FAD cofactor. From there, the electron is passed to a haem group, which donates it to O_2 on the extracellular side of the membrane, generating $\text{O}_2^{\bullet-}$.³⁰ Depending on the specific NADPH oxidase expressed in different cells, they can trigger different cellular transformations with widely differing biological outcomes. The NADPH oxidase family of enzymes illustrates the specificity in ROS generation and its impact on normal cellular signaling and homeostasis.³⁰

Mitochondria represent another major source for intracellular ROS production. The production of mitochondrial superoxide radicals occurs primarily at two discrete points in the electron-transport chain, namely at Complex I (NADH dehydrogenase) and at Complex III (ubiquinone-cytochrome *c* reductase) upon one electron transfer to oxygen.^{31,32} *In vitro*, these two sites in mitochondria convert 1–2% of the consumed oxygen molecules into superoxide anions both under normobaric or hyperbaric conditions.^{33–35} These initial estimates were made on isolated mitochondria and it may be concluded that the *in vivo* rate of mitochondrial superoxide production is considerably less.¹⁰ Although one-electron reactions predominate, two-electron reactions that allow the direct reduction of molecular oxygen to hydrogen peroxide do exist within the mitochondria.³⁶ Superoxide produced at Complex I is thought to form only within the matrix, whereas at Complex III superoxide is released both into the matrix and the inner mitochondrial space (IMS). A nonenzymatic source of ROS in mitochondria is the formation of the free radical semiquinone anion species ($Q^{\bullet-}$) that occurs as an intermediate in the redox cycling of coenzyme Q_{10} .¹⁰ Once formed, $Q^{\bullet-}$ can readily transfer electrons to molecular oxygen with the subsequent generation of a superoxide radical. The generation of ROS therefore becomes predominantly a function of metabolic rate.

In addition to the mitochondria and NADPH oxidases, other cellular sources of ROS production include a number of intracellular enzymes such as the flavoenzyme ERO1 in the endoplasmic reticulum, xanthine oxidase, cyclo-oxygenases, cytochrome p450 enzymes, lipoxygenases, flavin-dependent demethylase, oxidases for polyamines and amino acids, and nitric oxide synthases that produce oxidants as part of their normal enzymatic function.^{30,32} Free copper ions or iron ions that are released from iron-sulfur clusters, haem groups or metal-storage proteins can convert $O_2^{\bullet-}$ and/or H_2O_2 to $OH^{\bullet}$ in what is known as the Fenton reaction (Scheme 1.2).^{37–39} A similar reaction but involving lipid hydroperoxides accounts for the formation of lipid alkoxyl ($LO^{\bullet}$) and peroxy radicals ($LOO^{\bullet}$) in the lipid membrane.⁷

We next briefly mention biological sources of singlet oxygen, discussed in the following chapters. A possible pathway of cellular singlet oxygen formation is from oxygen in areas of inflammation through the action of Phox (NOX of phagocytes mainly in neutrophils and macrophages) and the oxidation of halide ions by the phagocyte enzyme myeloperoxidase (MPO).²⁹ Additionally, superoxide and NO are readily converted by enzymes or nonenzymic chemical reactions into reactive nonradical species amongst which are singlet oxygen, hydrogen peroxide, or peroxynitrite ($ONOO_2$).¹²



Scheme 1.2. Fenton reaction.

1.3.2. Commonly Encountered Extrinsic Sources of ROS

Reactive oxygen species can be produced by a host of exogenous processes. Environmental sources include ultraviolet light, ionizing radiation, and pollutants. Amongst the pollutants are chemicals (*e.g.* paraquat, also named methyl viologen) that react to form either peroxides or ozone; chemicals that promote the formation of superoxide such as quinones, nitroaromatics, and bipyrimidiulium herbicides (related to paraquat); chemicals that are metabolized to radicals, *e.g.*, polyhalogenated alkanes, phenols, aminophenols; or chemicals that release iron and copper that could promote the formation of hydroxyl radicals.^{8–10}

ROS could be generated rapidly through radiolysis of water molecules upon ionizing radiation (X-rays, γ -rays) or UV-light irradiation of H_2O_2 .⁴⁰ Secondary ROS products generated through this method can potentially amplify the initial ionization event. However, theoretical calculations show that hydrogen peroxide or superoxide anion are generated in very low concentrations by the primary ionization event.⁴⁰ In the presence of a sensitizer UV radiation could additionally lead to the formation of singlet oxygen, amply discussed in this book.

Environmental agents including non-DNA reactive carcinogens can generate ROS in cells by metabolism to primary radical intermediates or by activating endogenous sources of reactive oxygen species.⁴¹ The induction of oxidative stress and damage has been observed following exposure to xenobiotics of varied structures and activities. Chlorinated compounds, radiation, metal ions, barbiturates, phorbol esters, and some peroxisome-proliferating compounds are among the classes of compounds that induce oxidative stress and damage *in vitro* and *in vivo*.⁴¹

The mechanism of action of many chemotherapeutic cancer drugs involves ROS-mediated apoptosis. For example, the classic antitumor drugs cisplatin and adriamycin appear to produce ROS at excessive levels, resulting in DNA damage and cell death.⁴² Some classes of antibiotics rely on a similar mechanism for their bactericidal activity. For example, it was recently shown that bactericidal antibiotics, regardless of drug–target interaction, induce a breakdown in iron regulatory dynamics, stimulating the production of highly deleterious hydroxyl radicals through Fenton reaction in gram-negative and gram-positive bacteria, which ultimately contribute to cell death.⁴³

1.4. Chemical Reactivity of ROS

ROS are oxidant species that can operate *via* one-electron oxidation (radical ROS species) or two-electron oxidation (nonradical ROS species).⁷ In the former case reactivity is strongly linked to thermodynamics as activation barriers for the one-electron reaction of radicals are expected to be low.⁸ One may then utilize Table 1.1 in estimating the reactivity for different ROS. Radical ROS species are typically initiators or chain propagators in chain reactions; a notable example is the free radical-mediated auto-oxidation of polyunsaturated fatty acids (PUFA) that relies on lipid peroxy radicals as chain propagators.^{44–50}

The reactivity of nonradical ROS species will on the other hand be strongly dependent on the activation barrier to the reaction of interest. Based on a scale of reactions with glutathione (GSH) one may observe that HOCl is more reactive than H_2O_2 (rate constants of $3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $0.9 \text{ M}^{-1} \text{ s}^{-1}$, respectively) albeit the redox potential for the 2-electron reduction is larger for the latter in forming water, than for the former in forming chloride.⁸

1.4.1. Biological Targets of ROS

The high toxicity associated to ROS would imply that they are indiscriminate in choosing biological targets to react with, yet a closer look to their now accepted signaling role in cells would rather point to a well-orchestrated target choice. This paradox may be reconciled if one realizes the broad range of reactivities for the various species contained within the ROS family. Highly reactive ROS (e.g. $\text{OH}^\bullet$) will not be selective and will have a broad range of nonspecific targets. They will further have extremely short lifetimes in solution. ROS characterized by a relatively low reactivity, such as H_2O_2 or $\text{O}_2^{\bullet -}$, will in turn be relatively selective. Their chemical activity towards different substrates in competing reactions will be dictated by the interplay of substrate relative availability and relative rate constants of reaction.

Signaling by – low-reactivity – ROS involves reaction with only a few atomic elements within target macromolecules, and frequently with only a subset of these atoms within a given macromolecule, leading to covalent protein modification.²⁶ Accordingly, we may first discuss the *atomic targets* of ROS to then address *molecular targets*.²¹

Atomic targets: reaction of ROS with sulfur, found in methionine and cysteine is typically favored; also selenium found in seleno-cysteine is a commonly encountered ROS atomic target.³⁰ In both cases and given their redox potentials, reactions may be reversible. Another common atomic target is carbon either in nucleosides, or in aminoacids such as arginine, lysine, proline and threonine,⁵ as well as carbon in polyunsaturated fatty acids.⁴⁶ An additional element targeted by ROS is iron, where ROS may react with [Fe–S] clusters²² and with iron within haem.

Hydrogen peroxide typically reacts with thiols in their anionic form, thus thiols with low pK_a are found to be more reactive. The solution pH may further tune the reactivity towards this ROS.^{26,51–54} Within a specific protein, and given the range of pK_a different thios may have, as a result of close proximity to other functional groups, only a subset of the thiols may be involved in reaction with H_2O_2 . This leads to very specific response to exposure to this ROS.⁵⁵ Rate constants of reactions may range from $2 \times 10^1 \text{ M}^{-1} \text{ s}^{-1}$ for free Cys to $1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for specific Cys residues within a protein.²⁶ Rate constant of reactions of H_2O_2 with [Fe–S] clusters in turn are relatively small, *ca.* 1×10^2 to $1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$.²⁶

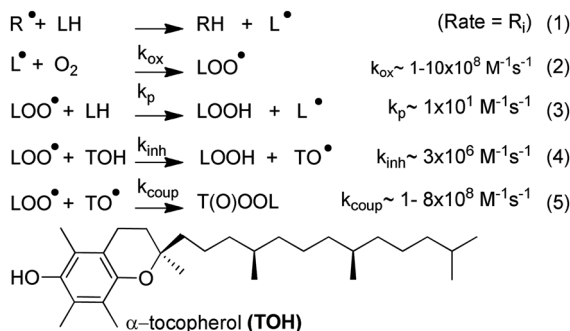
Superoxide radical anion in turn favors reaction with [Fe–S] clusters (it can achieve diffusion-controlled rates)²⁶ where the vulnerability of these groups is partly due to the favorable electrostatic interactions with $\text{O}_2^{\bullet -}$.²² The relative

reactivities of H_2O_2 and $\text{O}_2^{\cdot-}$ lead to preferred biological targets, exemplified by transcription factors SoxR ([Fe-S] cluster) and OxyR (Cys residue) in *Escherichia Coli*.^{56,57} These factors are activated by $\text{O}_2^{\cdot-}$ and H_2O_2 , respectively, and they are involved in the expression of antioxidant enzymes. Here, SoxR regulates responses to $\text{O}_2^{\cdot-}$,⁵⁸ and OxyR regulate responses to H_2O_2 .^{59,60}

Molecular targets, proteins: a large number of proteins are affected by ROS, where following ROS attack conformational changes take place that regulate protein activity. This is best exemplified by the ever-increasing⁶¹ list of proteins where Cys residues act as redox switches. Here, disulfide bond formation following oxidation of Cys residues may result in structural and associated activity changes.^{59,62} Cys residue oxidation in phosphatases is an important target in biological systems as it affects protein phosphorylation and thus has a broad impact in the cell proteome. Oxidative stress in proteins leads to formation of carbonyl derivatives along their backbone, used as markers of general oxidative stress.^{10,63}

Molecular targets, DNA: mitochondrial DNA is a major target of ROS given that mitochondria are the prevalent source of ROS within cells. This leads to compromised mitochondrial function. ROS reactions with DNA itself, rather than proteins, may serve to promote transcription.³⁰ Even if reactions with DNA may be a negligible part of the ROS reactions within cells, their impact is far reaching.⁸ Aging cells have an increased level of ROS-damaged nuclear DNA.¹⁰

Molecular targets, lipids: a significant body of work, both in model membrane systems and in live cells, has examined the role lipid peroxyl radicals play in damaging the cell lipid milieu. Autooxidation of polyunsaturated fatty acid residues is initiated by a free radical such as the hydroxyl radical, which upon reaction with fatty acids generate lipid carbon centered radicals (eqn (1.1), Scheme 1.3).^{44,47,64-66} Lipid carbon centered radicals in turn readily trap molecular oxygen under physiological conditions to form lipid peroxyl radicals,^{67,68} effective chain carriers in the lipid chain auto-oxidation (eqn (1.2) and (1.3), Scheme 1.3). In the oxidation process, fatty acyl chains mostly in their *cis* configuration are either converted to the *trans* configuration,^{44,69-71}



Scheme 1.3. Lipid (L) oxidation in the presence of a free-radical initiator ($\text{R}^{\cdot}$) and α -tocopherol (TOH); eqn (1.2),⁶⁷ (1.3),⁶⁸ (1.4)⁷⁷ and (1.5).⁷⁸

or form corresponding hydroperoxides and alcohols^{44,72} or may fragment into electrophilic $\alpha\beta$ -unsaturated aldehydes,^{72,73} among others. Peroxidation and destruction of the *cis* double bonds may in turn lead to a reduction in the membrane fluidity⁷⁴ and appearance of liquid-order domains.⁷⁵ Auto-oxidation of polyunsaturated fatty acid residues ultimately generates a variety of secondary cytotoxic products that account for pathological effects, *e.g.*, neurodegenerative diseases,¹³ atherosclerosis,⁴⁹ and cell apoptosis.⁷⁶ Importantly, polyunsaturated fatty acids within the inner mitochondrial membrane are particularly vulnerable to ROS elicited oxidative damage.¹³

Oxidative signaling pathways arise from the formation of electrophilic $\alpha\beta$ -unsaturated aldehydes that may undergo reaction with nucleotides (indirect signaling).²⁰ Additional oxidative signaling pathways have been reported that involve cardiolipin peroxidation and release of proapoptotic factors from mitochondria,⁷⁹ as well as phosphatidyl serine (PS) oxidation in the plasma membrane leading to externalization and recognition of PS on the cell surface by phagocytes.⁷⁶

1.4.2. ROS Scavengers and Antioxidants

Enzymatic and nonenzymatic antioxidant systems in cells regulate the concentration of ROS. Notably, McCord and Fridovich in the late 1960s discovered a variety of enzymes that were found to be responsible for detoxification of oxygen in aerobes but were absent in anaerobes (leading to oxygen-induced damage in these organisms).^{80,81} The finding of superoxide dismutase (SOD) was a landmark discovery in the field of free-radical biology.⁸² The presence of such enzymes suggested that if the ROS were not scavenged, they would critically injure cells. SOD ensures that the level of $O_2^{\cdot -}$ remains below 0.1 nM in *E. coli*.^{39,83} Over the years, new examples of enzymatic antioxidant systems have emerged. They include catalase, and glutathione and NADH peroxidase. The latter ensure that the steady-state concentration of H_2O_2 within *E. coli* does not exceed 20 nM.³⁹

Examples of nonenzymatic antioxidants include glutathione, vitamin C (both water soluble) and α -tocopherol (lipid soluble, see also Scheme 1.3).^{2,10,84} All three antioxidants scavenge free radicals. Ascorbic acid yields ascorbyl radicals that readily disproportionate so no secondary free-radical byproducts are formed. In the case of glutathione, the thyl radical formed may be a concern as it may react with lipids and proteins yet it readily forms a disulfide bond with the thiolate of a second glutathione molecule. The disulfide radical anion is next scavenged by oxygen yielding an inert disulfide.⁸

A member of the vitamin E family of compounds, α -tocopherol (TOH) has long been recognized as the most active naturally occurring lipid soluble antioxidant (Scheme 1.1 and Figure 1.1).^{77,85} The paradigm of TOH antioxidant activity in auto-oxidation reactions has been laid out in a number of studies conducted over the past 30 years in homogeneous solution and in the presence of initiators (see Scheme 1.3, reactions 1.4 and 1.5).

In a first elementary step TOH reacts with a peroxy radical ($\text{ROO}^\bullet$ or $\text{LOO}^\bullet$) *via* H-atom transfer to yield a tocopheroxyl radical ($\text{TO}^\bullet$, eqn (1.5)) and a hydroperoxide ROOH/LOOH . Following coupling of $\text{TO}^\bullet$ to a second peroxy radical a second chain-termination reaction occurs. TOH effectively terminates two chain reactions.⁷⁶ The tocopheroxyl radical may also be scavenged by ascorbate at the lipid water interface where ascorbate acts as the ultimate ROS sink.⁷⁶

1.4.3. ROS Lifetime and Diffusion

An interesting discussion is how far a given ROS will diffuse on average before decay through a unimolecular process or upon scavenging by, *e.g.* an antioxidant or a target molecule. Given their low concentration in biological tissue, second order reactions involving encounter of 2 identical ROS such as lipid peroxy radicals are rare. Mostly ROS decay *via* first-order or pseudofirst-order reactions (*e.g.* upon scavenging by ascorbate, glutathione, or an enzyme). The average lifetime τ (inverse of the experimental decay rate constant k_{exp}) may thus be obtained for a given ROS given the rate constant of unimolecular reaction (k^0), the rate constant of reaction with scavenger (k_{qs}), and abundance of scavengers ($[\text{S}]$) (eqn (1.1)). In turn, one may next assume a freely diffusing ROS molecule (generally applicable for noncharged ROS) with an average diffusion coefficient D of $1 \times 10^{-5} \text{ cm}^2$ (average value for a small molecule in water) and estimate *via* eqn (1.2) the mean square displacement $\langle r^2 \rangle^{1/2}$ utilizing the average lifetime. Pryor⁸⁶ and Winterbourn⁸ provide relevant numbers to estimate the mean square displacement for a number of ROS. While $\text{OH}^\bullet$ is scavenged within a few angstroms of its generation site, $\text{O}_2^{\bullet -}$ and H_2O_2 may diffuse a few tens of micrometers (albeit $\text{O}_2^{\bullet -}$ is charged and may not readily cross bilayers) thus exerting a long-range effect.

$$\tau_{\text{exp}} = \frac{1}{k_{\text{exp}}} = \frac{1}{k^0 + k_{\text{qs}} \times [\text{S}]}, \quad (1.1)$$

$$\sqrt{\langle r^2 \rangle} = \sqrt{6 \times D \times \tau_{\text{exp}}}. \quad (1.2)$$

1.5. Monitoring ROS

Valuable methods to study the generation and evolution of ROS and associated chemical processes *in vitro* and *in vivo* include HPLC, mass spectrometry, EPR (when dealing with free radicals) and other analytical procedures that provide information on the biological production of ROS by detecting specific products generated from the oxidation of protein, DNA, lipids, or other biomolecules.^{46,87,88} These methods have the drawback of being generally destructive, some further lack the necessary sensitivity, and they may be limited to providing information on the products of reactions of ROS and not on the specific rate or location of ROS production.

1.5.1. Fluorogenic Probes

Advances in fluorescence microscopy have allowed for the development of noninvasive tools that provide high specificity to a ROS species and sensitivity combined with spatial and temporal resolution for imaging ROS evolution in live cells. The new probes enable monitoring ROS in biological systems and correlating their sites of production to important physiological processes.^{89–98} Specificity to a particular type of ROS is of high importance for the design of successful probes. The ideal molecular probe for ROS would also be highly reactive at low concentrations; sensitive; nontoxic; easy to load into organelles, cells, or tissues without subsequent leakage or unwanted diffusion, excretion, or metabolism.⁹⁹ It should further be able to identify the site of production of the oxidant, quantify the produced amount of ROS as well as provide information that will enable the mechanistic understanding of the disturbance in cellular redox state. Fluorescent probes developed in recent years cover the majority of these specifications, however quantitative evaluation of the ROS production both *in vitro* and *in vivo* still remains a challenge.¹⁰⁰

Most of the fluorescent probes developed to date for *in vitro* and *in vivo* imaging are designed to activate in the presence of the analyte of interest. The activation results most often in an increase in fluorescence (off/on fluorogenic probes) or a shift in the emission wavelength (ratiometric probes). Most of the widely used probes that are commercially available are prefluorescent aromatic molecules that undergo oxidation in the presence of ROS to a fluorescent product (Figure 1.3). Many of the newer probes developed

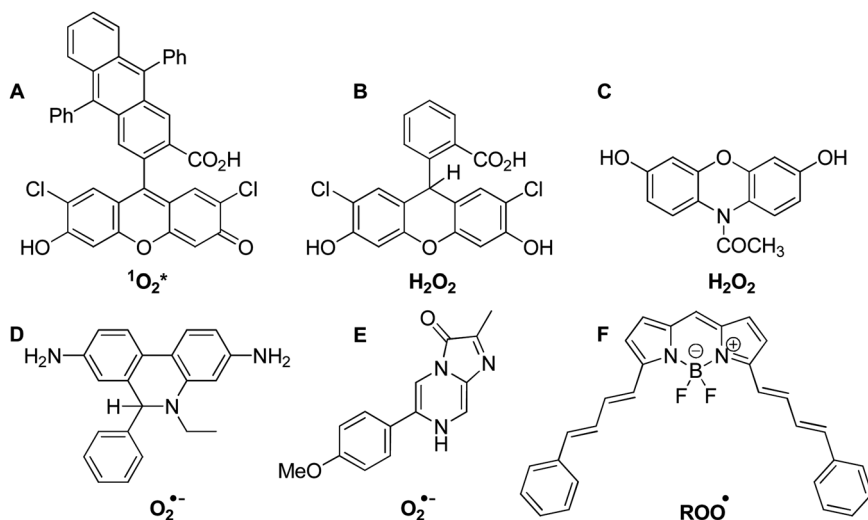


Figure 1.3. Commercial fluorogenic probes for sensing ROS which exert emission enhancement upon oxidation of the aromatic core: (A) DPAX probe developed by Nagano *et al.*^{89,103} (B) 2',7'-Dichlorodihydro-fluorescein (DCFH); (C) Amplex red; (D) hydroethidine; (E) MCLA (luciferin analog, 2-methyl-6-(4-methoxyphenyl)imidazo[1,2-a]pyrazin-3(7H)-one); (F) Bodipy® 665/676 (ratiometric probe; shift in emission wavelength is observed upon oxidation of the conjugated double bonds).

in recent years are compounds containing a masked fluorophore that is released by attack of the oxidant on the masking group (Figure 1.4). Probes that fall into the second category generally rely on photo-induced electron transfer (PeT) as the molecular switch of fluorescence. Deactivation of PeT upon oxidation of the trap segment restores the emission of the reporter segment. For more detailed information we would recommend several reviews published in recent years.^{93,99,101,102}

The complex biology of ROS is dictated not only by the chemical properties of each type of oxygen metabolite but also their production sites and further trafficking within the cell.¹¹ This provides a motivation for developing tools to study the chemistry and biology of ROS in specific organelles in the cell. Fluorescence imaging with fluorogenic probes that can target specific organelles emerges as a valuable method for site-specific sensing of the different types of ROS exploring their complex contributions to physiological processes in living organisms. Most advances in this field have been made by developing fluorogenic probes that preferentially target mitochondria and that react specifically with H_2O_2 ,^{106,107} superoxide,¹⁰⁸ lipid-based ROS,^{98,109} singlet oxygen,¹¹⁰ hypochlorous acid,¹¹¹ and highly reactive ROS.^{112,113}

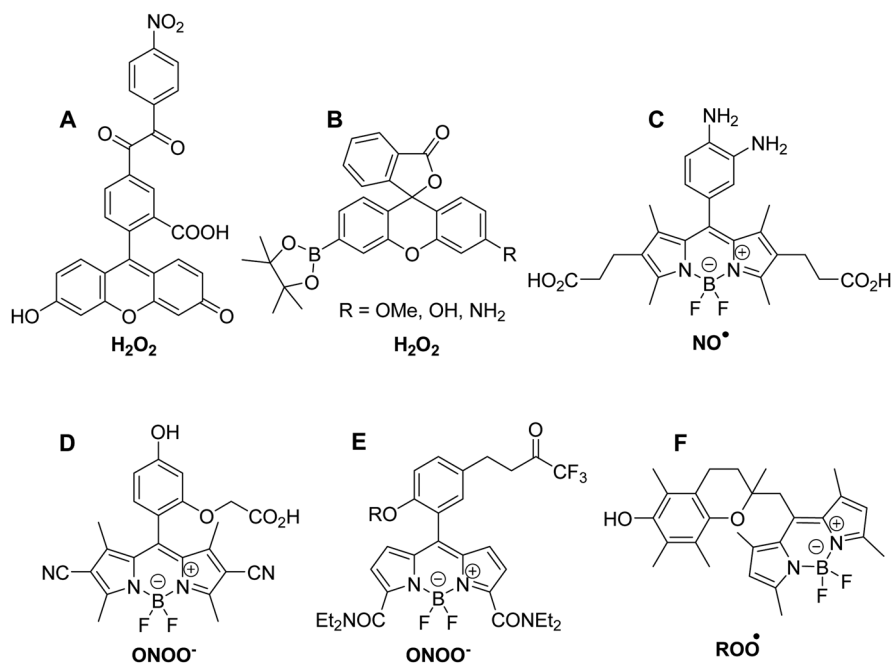


Figure 1.4. Fluorogenic probes for sensing ROS: (A) NBzF probe for hydrogen peroxide;¹⁰⁴ (B) hydrogen peroxide sensor (H_2O_2);⁹¹ (C) nitric oxide sensor DAMBO-PH;⁹² (D) peroxynitrite sensor NiSPY-3;⁹⁴ (E) peroxynitrite probe HK-Green;¹⁰⁵ (F) H_2B -PMHC probe for detection of lipid peroxyl radicals.⁹⁶

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Chapter 2

Properties of Singlet Oxygen

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2.1. Introduction: Ground and Excited States of Molecular Oxygen

Molecular oxygen is a diatomic homonuclear molecule that, despite its apparent simplicity, exhibits rather unusual properties with respect to its magnetic behavior, spectroscopy, energy-transfer processes and chemical reactivity. These singularities come as a result of the particular electronic configuration of its ground and excited states.

The electronic configuration of O_2 in its ground state is $(1\sigma_g)^2(1\sigma_u)^2(2\sigma_g)^2(2\sigma_u)^2(3\sigma_g)^2(3\sigma_u)^2(3\pi_g)^4(3\pi_u)^2$. Unlike other homonuclear molecules, ground-state molecular oxygen has an open-shell electronic configuration with two unpaired electrons that, according to Hund's rule, occupy different molecular orbitals (Figure 2.1(A)). This electronic configuration is commonly designated with the terminology $O_2(^3\Sigma_g^-)$ or $^3O_2$; the superscript "3" indicates that it corresponds to a triplet state, the " Σ ", that its orbital angular momentum (M_L) equals 0; and the subscript "g", that the symmetry of the molecule is pair (g from the German *gerade*). The two lowest-energy excited states of oxygen are both spin singlet states and have pair parity, but differ in M_L (Figure 2.1(A)). The lowest-energy singlet excited state, $O_2(^1\Delta_g)$ or $^1O_2$, has an M_L equal to 2, depicted with the Δ symbol. On the other hand, the M_L of the next higher-energy singlet excited state is 0, being another sigma state, $O_2(^1\Sigma_g^+)$.¹

In this chapter we will review the thermodynamic properties of $^1O_2$ as well as its physical deactivation mechanisms, going from homogeneous systems, *i.e.* gas phase and solution state, to more challenging heterogeneous systems including polymers, proteins and cells.

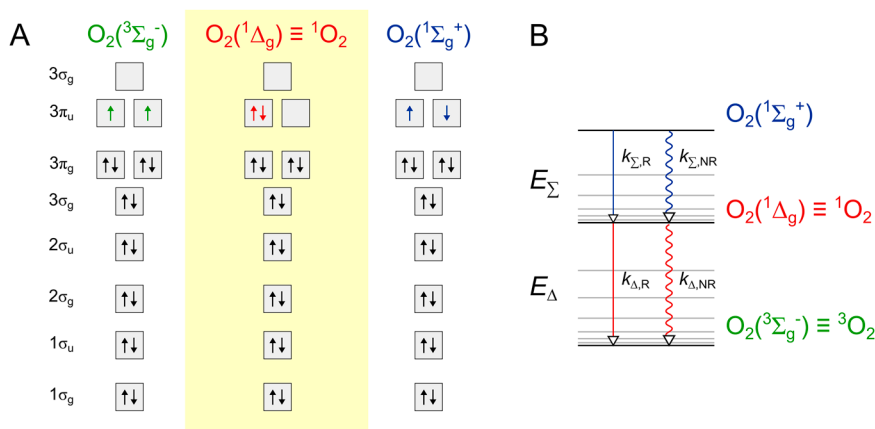


Figure 2.1. (A) Electronic configuration of ground state molecular oxygen $O_2(^3\Sigma_g^-)$, its first singlet excited state, $O_2(^1\Delta_g)$ namely singlet oxygen $^1O_2$, and its higher-energy singlet state, $O_2(^1\Sigma_g^+)$. (B) Jablonsky diagram of molecular oxygen and its first singlet excited states.

2.2. Thermodynamic and Electrochemical Properties of Singlet Oxygen

The first excited state of molecular oxygen, $^1\text{O}_2$, lies 94.3 kJ mol^{-1} (E_{Δ}) above the ground state and O_2 ($^1\Sigma_g^+$) is 63 kJ mol^{-1} higher in energy than the former (E_{Σ} , Figure 2.1(B)).² On the basis of these energy levels, luminescence decays of excited singlet states of oxygen are detected at 1270 nm ($^1\text{O}_2 \rightarrow ^3\text{O}_2 + h\nu$) and at 762 nm (O_2 ($^1\Sigma_g^+$) $\rightarrow ^3\text{O}_2 + h\nu$).² Frequently, the excited-state energy of organic molecules is comparable to classic bond-dissociation energies, *i.e.* in the range of $165\text{--}400 \text{ kJ mol}^{-1}$ and thus it comes as no surprise to find bond rupture as a regular consequence of excited-state processes. On the contrary, the energy of $^1\text{O}_2$ is lower to that of an ordinary chemical bond and thus one anticipates that bond cleavages will not be plausible reactions of $^1\text{O}_2$ chemistry unless they are accompanied by other changes that compensate for the energy requirements (see Chapter 20).

As a result of its high electronegativity, oxygen is an excellent electron acceptor and a very poor electron donor. The addition of an electron to O_2 leads to different reactive oxygen species (ROS) such as $\text{O}_2^{\cdot-}$, $\text{OH}^{\cdot}$, $\text{HO}_2^{\cdot}$ or H_2O_2 , which are strong oxidants. The rate-limiting step for the formation of all these species is the first electron-transfer reaction (et) to O_2 (eqn (2.1)).



In its ground state, the redox couple $\text{O}_2/\text{O}_2^{\cdot-}$ has a reduction potential (E_{red}) of -0.15 V in water and -0.60 V in *N,N*-dimethylformamide (DMF), referenced to the standard hydrogen electrode (SHE).³ However, its electronic excited state, $^1\text{O}_2$, is a better oxidant and reductant than $^3\text{O}_2$ with E_{red} of 0.79 V in water and 0.34 V in DMF (referenced to SHE).³ The full et from donor molecules to $^1\text{O}_2$ was first described by Manring *et al.* for *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) in D_2O and later confirmed by Darmanyan *et al.*, resulting in the formation of $\text{O}_2^{\cdot-}$ and $\text{TMPD}^{\cdot+}$ exclusively in water but not in other protic solvents.^{4,5} More recently, the et to $^1\text{O}_2$ by metal complexes in aprotic solvents was described.⁶ However, the full et to $^1\text{O}_2$ is a rare situation. Generally, $^1\text{O}_2$ is deactivated in the presence of electron-donor groups such as amines or aromatic hydrocarbons through a charge-transfer (CT) exciplex $^1(\text{O}_2^{\delta-} \dots \text{donor}^{\delta+})$ in a nonradiative pathway.^{5,7}

2.3. Physical Mechanisms of Singlet Oxygen Deactivation

Undoubtedly, one of the major breakthroughs in the $^1\text{O}_2$ scientific community in the last 80 years has been the development of tools to directly monitor $^1\text{O}_2$ decay rates by its characteristic phosphorescence emission at 1275 nm in time-resolved experiments (see Chapter 20). This technique has allowed us to unequivocally assign the $^1\text{O}_2$ decay rate constant (k_{Δ}) or its reciprocal parameter, the $^1\text{O}_2$ lifetime (τ_{Δ}), in different media, revealing that $^1\text{O}_2$ decay kinetics is extremely dependent on the environment where $^1\text{O}_2$ is located.

The k_{Δ} is a first-order rate constant that reflects all the processes that contribute in $^1\text{O}_2$ removal for a given system and can thus be expressed as the sum of all the rates for each of the chemical and physical channels of deactivation (eqn (2.2)):

$$k_{\Delta} = \tau_{\Delta}^{-1} = k_{\Delta,R} + k_{\Delta,NR} + k_{\Delta,r}, \quad (2.2)$$

where $k_{\Delta,R}$ is the rate constant for the radiative $^1\text{O}_2$ deactivation, $k_{\Delta,NR}$ is the rate constant for the nonradiative $^1\text{O}_2$ deactivation and $k_{\Delta,r}$ is the rate constant for chemical reaction quenching of $^1\text{O}_2$.

2.3.1. Radiative Deactivation of Singlet Oxygen

The *pure* radiative lifetime of the $^1\text{O}_2 \rightarrow ^3\text{O}_2 + h\nu$ transition ($\tau_{\Delta,R}$) is exceptionally long, $\approx 2700 \text{ s}^8$ as it is a forbidden electric dipole process in terms of spin, parity and angular momentum. However, at higher gas-phase densities such as in pure oxygen the transition probability of $^1\text{O}_2$ increases dramatically, resulting in smaller $\tau_{\Delta,R}$.⁹ Similar results are observed in solution where $\tau_{\Delta,R}$ varies from few microseconds to several nanoseconds.^{10–12}

The effect of solvent on $\tau_{\Delta,R}$ has been the subject of intense experimental investigation as the intensity of the $^1\text{O}_2$ phosphorescence depends on both the $^1\text{O}_2$ production quantum yield (Φ_{Δ}) and $k_{\Delta,R}$ ($\tau_{\Delta,R}^{-1}$).¹³ Clearly, this is an important problem from a practical perspective since $^1\text{O}_2$ phosphorescence intensity is often used to obtain and compare Φ_{Δ} values of a variety of photosensitizers (PSs) in different solvents.¹⁴ The strong variation of $\tau_{\Delta,R}$ in different solvents limits the comparison of the $^1\text{O}_2$ signals for both sample and reference in the same solvent. Moreover, the previous difficulty is significantly increased in heterogeneous systems, particularly in polymers,¹⁵ proteins^{16,17} and cells, as the microenvironment where $^1\text{O}_2$ is produced is considerably heterogeneous.

Efforts to compare the variation of $k_{\Delta,R}$ with specific solvent parameters have resulted in some correlations. First, there is a reasonable correlation between $k_{\Delta,R}$ and the solvent polarizability expressed as a function of the solvent refractive index, according to which $k_{\Delta,R}$ is expected to increase with the polarizability of the solvent.^{10–12} Secondly, poor correlation between $k_{\Delta,R}$ and the solvent ionization potential has been found, indicating that a charge-transfer character between a solvent- $^1\text{O}_2$ complex is not likely to be the principal factor for the modification of the transition probability.¹² Finally, $k_{\Delta,R}$ is proportional to the square of the molar refraction of the perturbing molecule.¹⁸

These observations were initially explained in agreement with the formation of a bimolecular (1:1) collision complex between $^1\text{O}_2$ and a perturbing molecule that gave rise to a new set of molecular eigenfunctions that partially allowed its deactivation (Figure 2.2).^{9,18,19} However, the analysis of the solvent effect on the radiative decay of $^1\text{O}_2$, including both the $\tau_{\Delta,R}$ and the wavelength of the maximum for the $^1\text{O}_2 \rightarrow ^3\text{O}_2 + h\nu$ transition, suggested

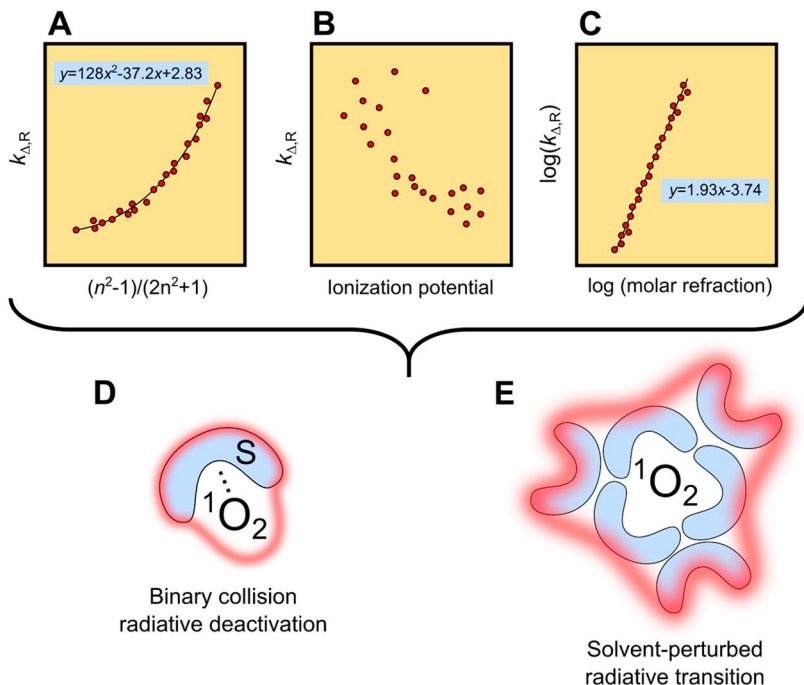


Figure 2.2. Schematic representation of the experimental evidence (A),¹² (B)¹² and (C)¹⁸ that supports the two main theories, the binary collision radiative deactivation (D) and the solvent-perturbed radiative transition (E) for the $^1O_2$ solvent-induced radiative deactivation.

that the Kirkwood–Onsager reaction field model may provide an alternative approach to interpret the solvent-perturbed radiative transition.¹² According to this model, oxygen is perceived to exist in a cavity defined by the solvent, which is considered to be homogeneous and isotropic with defined macroscopic constants (Figure 2.2). The latter model has been recently supported by Jensen *et al.* when studying the temperature effects on $\tau_{\Delta,R}$.²⁰

2.3.2. Nonradiative Deactivation of Singlet Oxygen

Apart from chemical reactions, three types of interactions contribute to the nonradiative deactivation of $^1O_2$:²¹ (a) electronic energy transfer to solute molecules such as carotenoids,^{22,23} (b) charge-transfer quenching^{5,7,24} and (c) electronic to vibrational energy transfer. All previous processes are described with bimolecular rate constants as they require collisions between $^1O_2$ and a solute or solvent molecule. Therefore, the actual value of the unimolecular rate constant $k_{\Delta,NR}$ obeys the following expression (eqn (2.3)):

$$k_{\Delta,NR} = k_{\Delta,ET}[Q_{ET}] + k_{\Delta,CT}[Q_{CT}] + k_{\Delta,e-v}[S], \quad (2.3)$$

where $k_{\Delta,ET}$ is the rate constant for the energy transfer quenching, $k_{\Delta,CT}$ is the rate constant for the charge-transfer deactivation, $k_{\Delta,e-v}$ is the rate constant for electronic to vibrational energy transfer, $[Q_i]$ is the concentration of each quencher and $[S]$ the concentration of solvent.

From all the above deactivation pathways, the main one responsible for the characteristic τ_{Δ} in homogeneous solutions is the electronic to vibrational energy-transfer deactivation. In order to undergo electronic to vibrational energy-transfer deactivation, $^1O_2$ must transfer its energy to a vibrational mode of the solvent. The better the matching between the energy of this vibrational mode and that of the $^1O_2$ deactivation energy, the faster this process occurs (Figure 2.3). Hence, the deactivation of $^1O_2$ is favored in O–H containing solvents, *e.g.* H_2O , whereas it is clearly reduced in C–H and specially, C–F comprising solvents (Table 2.1).²⁵ Similarly, deuterated solvents have lower matching with the $^1O_2$ vibrational deactivation, and therefore the lifetimes are considerably increased in such media (Table 2.1). Typically, the ratio between the rate constants for $^1O_2$ decays in nondeuterated and deuterated media ($k_{\Delta}^H/k_{\Delta}^D$) varies from 10 to 30.^{26,27} Moreover, in nonaromatic

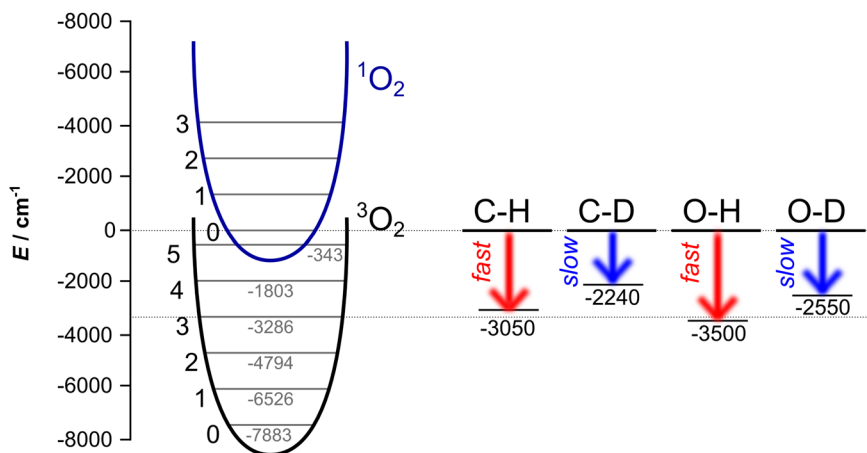


Figure 2.3. Comparison of the energy levels of $^1O_2$ to common high-frequency X–H and X–D vibrations of solvents.

Table 2.1. Rate constants of the electronic to vibrational energy transfer of $^1O_2$ to different X–Y bonds in solution.^{27,28}

X–Y	$k_{\Delta,NR}^{X-Y}, M^{-1} s^{-1}$
O–H	2900
N–H	1530
C–H	309
O–D	132
C–D	10.4
C–Cl	0.181
C–F	0.049

solvents, Rodgers²⁷ found that the value of $k_{\Delta, \text{NR}}$ attributed to the electronic to vibrational energy transfer can be estimated by the addition of individual factors of the various atomics groups (X-Y): -CH-, -CH₂-, -OH, -OD... ($k_{\Delta, \text{NR}}^{\text{X-Y}}$, eqn (2.4))

$$k_{\Delta, \text{NR}}^{\text{e-v}} = \sum_i N_i k_{\Delta, \text{NR}}^{\text{X-Y}}. \quad (2.4)$$

2.4. Singlet Oxygen Diffusion

Once ¹O₂ is produced it can diffuse through the surrounding medium. The distance traveled by ¹O₂, d , depends on the magnitude of the diffusion coefficient (D) and on τ_{Δ} . Assuming a radial diffusion over time (t), the distance traveled by ¹O₂ can be expressed as eqn (2.5), deduced from Fick's law for a three-dimensional diffusion of a molecule in a uniform concentration gradient.²⁹

$$d = \sqrt{6Dt}. \quad (2.5)$$

The value of t has been the subject of intense debate as it depends on the environment where ¹O₂ decays (Figure 2.4). In homogeneous solution, where the population of ¹O₂ is reduced by a factor of $1/e$ over time, it is reasonable to consider $t = 5\tau_{\Delta}$ because even after a time t 3- or 4-fold larger than τ_{Δ} there is still a non-negligible amount of ¹O₂ remaining.³⁰ On the other hand, in heterogeneous environments, one may take t as τ_{Δ} ,³¹ $3\tau_{\Delta}$ ³² or even $5\tau_{\Delta}$.³⁰ The former reduction is supported on the basis that the presence of natural quenchers as well as local diffusion domains might reduce the radial diffusion of ¹O₂.

A second parameter that defines the diffusion distance of ¹O₂ is D . As derived from the Einstein-Smoluchowski relation (eqn (2.6)), D is proportional to the temperature (T) and inversely proportional to the size of the

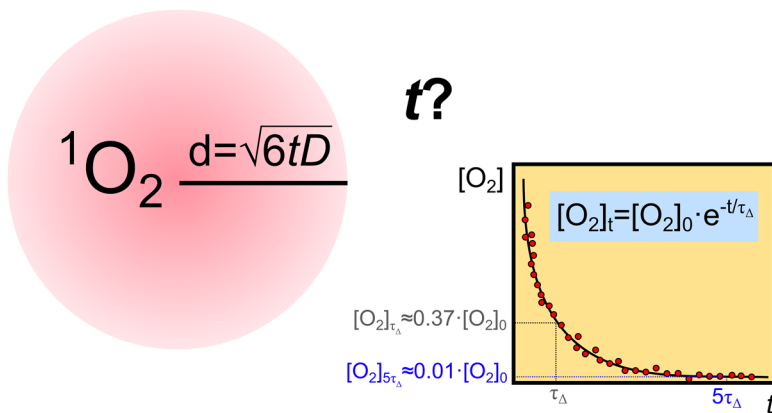


Figure 2.4. Schematic illustration for the calculation of the radial diffusion of ¹O₂.

diffusing molecule (r) and the viscosity of the media where it diffuses (η).²⁹ D has been characterized for a variety of solvents with different viscosities and at different temperatures such as air ($D = 0.232 \text{ cm}^2 \text{ s}^{-1}$, 298 K),³³ water ($D = 2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, 297 K),³⁴ D_2O ($D = 1.41 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, 294 K),³⁴ and ethanol ($D = 2.64 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, 303 K).²⁸ One should note that D is strongly reduced from gas to liquid phase as opposed to the viscosity.

$$D = \frac{k_B T}{6\pi r \eta}. \quad (2.6)$$

More challenging is the unequivocal identification of D in heterogeneous media, particularly in cells, as oxygen can preferably localize in cellular compartments where it is more soluble, *i.e.* lipid membranes, and thus show different D for each microenvironment. For a more detailed explanation see Section 2.5.3.

2.5. Singlet Oxygen Deactivation in Different Environments

2.5.1. Homogeneous Environments

The production and deactivation of $^1\text{O}_2$ in homogeneous solutions has been widely studied and described for the last 20–30 years. As the value of τ_Δ varies from solvent to solvent, a plethora of τ_Δ have been reported so far (Table 2.2) which have been collected in various extensive reviews.^{2,21,28}

Table 2.2. Singlet oxygen lifetime in various solvents.

Solvent	$\tau_\Delta/\mu\text{s}$	References
H_2O	3.3 ± 0.5	2,21,28,35,36
D_2O	66 ± 2	21,25,28,35–38
CH_3OH	9.9 ± 0.5	21,28,35–37
CD_3OD	240 ± 10	25,35,37
$\text{CH}_3\text{CH}_2\text{OH}$	15.5 ± 3.5	2,21,36,37
$(\text{CH}_3)_2\text{CO}$	50.5 ± 4	10,28,36–39
$(\text{CD}_3)_2\text{CO}$	750 ± 80	2,25,28,36,38,39
CH_2Cl_2	101 ± 39	2,21,28,38
CHCl_3	235 ± 30	2,21,28,38
CDCl_3	650 ± 150	2,21,25,28,38
CCl_4	$73\,000 \pm 14\,000$	2,21,28,38
CH_3CN	66.7 ± 13.5	2,10,21,28,38,39
Pentane	29 ± 6	2 and 28
THF	23 ± 2.6	2,10,21,38
DMSO	24.6 ± 5.4	2 and 21
Pyridine	37 ± 20	2 and 21
Hexane	30 ± 1	2 and 28
Cyclohexane	21 ± 4	2,10,21
CS_2	$37\,000 \pm 8000$	2 and 10
Benzene, C_6H_6	32 ± 3.3	2,10,21,25,28,35,38,39
$\text{C}_6\text{H}_5\text{Br}$	96 ± 53	2,21,25,38
C_6F_6	$12\,000 \pm 8000$	2,21,25,38
C_6D_6	665 ± 95	2,21,25,28,38,39
Toluene	27 ± 2	2,10,21,35

2.5.2. Polymeric Systems

Already in the late 1970s, it was proposed that $^1\text{O}_2$ was an important contributor to thermal and photochemical degradation of synthetic organic polymers.⁴⁰ Since then, some publications examining $^1\text{O}_2$ properties in films of the most common organic polymers have appeared.

The first report by Turro *et al.* in 1978 evaluated $^1\text{O}_2$ lifetime from the length of diffusion of oxygen in two standard polymeric films, polystyrene and polymethylmethacrylate (PMMA). It was an indirect method employing an endoperoxide solubilized in polymer films which was capable of generating $^1\text{O}_2$ thermally. In 1983 Byteva *et al.* were the first to detect the characteristic phosphorescence band of the $^1\text{O}_2 \rightarrow ^3\text{O}_2 + h\nu$ transition at 1270 nm in a steady-state experiment, pointing out that the photosensitizing mechanism appeared to be the same as in solution.

One year later, Lee and Rodgers reported a time-resolved experiment where $^1\text{O}_2$ was generated by 2-acetonaphthone in water-swollen Nafion membranes or powders. In particular, these systems were microheterogeneous due to the water pools embedded in the polymer matrix after water swelling. In all the cases studied $^1\text{O}_2$ decayed with a single exponential. Actually, $^1\text{O}_2$ had a lifetime of 55 μs for H_2O -swollen Nafion and 270 μs for D_2O -swollen Nafion. This fact, together with an observed $^1\text{O}_2$ lifetime between that in neat water and in neat perfluorocarbon, suggested that a kinetic model previously proposed by the same authors, describing the “apparent” or observed $^1\text{O}_2$ decay rate constant in microheterogeneous systems, may hold true for the swollen polymer.⁴¹ Since its first report, this model has long found application in numerous studies to describe $^1\text{O}_2$ lifetime in heterogeneous systems, such as liposome suspensions or even cells (*vide infra*). This model (eqn (2.7)) describes a distribution of $^1\text{O}_2$ during its natural lifetime between the two different phases composing the microheterogeneous system, in this particular case being the water-filled cavities and the fluorocarbon regions. The entrance and exit constants between the two phases are much larger than the $^1\text{O}_2$ decay rate constants within the two individual phases, and hence an overall rate constant, k_Δ is observed for the system:

$$k_\Delta = \frac{K_{\text{eq}} \times f_m \times k_{\text{int}} + (1 - f_m) \times k_{\text{ext}}}{K_{\text{eq}} \times f_m + (1 - f_m)}, \quad (2.7)$$

where k_Δ is the observed $^1\text{O}_2$ decay rate constant; K_{eq} is the equilibrium constant of $^1\text{O}_2$ between the two compartments or phases, expressed as the concentration of $^1\text{O}_2$ in the internal phase divided by that in the external phase; f_m is the volume fraction occupied by the internal phase and k_{int} and k_{ext} are the $^1\text{O}_2$ decay rate constants within the internal and the external phases, respectively. The validity of this model was indicative of a free distribution of $^1\text{O}_2$ in the two phases. In addition, it enabled calculation of the $^1\text{O}_2$ lifetime in the fluorocarbon matrix of Nafion, which yielded 360 μs . The experimental value of $^1\text{O}_2$ lifetime in vacuum-dried Nafion was 320 μs ,

corroborating the applicability of the model in this system. With this value and assuming the oxygen diffusion coefficient D to be $1 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ in Nafion, an estimation of a diffusion radial distance (eqn (2.5)) of 44 nm was obtained.

In 1989, Clough *et al.*⁴² reported a study in which time-resolved $^1\text{O}_2$ phosphorescence and absorption of the triplet-state species of a PS (acridine or phenazine) incorporated in the polymer matrix were analyzed as a function of temperature, matrix rigidity, copolymer composition or ambient oxygen partial pressure. The main polymers studied were polystyrene, PMMA, and a range of copolymers of methyl methacrylate (MMA) and ethyl acrylate (EA) of varying compositions. Unlike in liquid solution, in these glassy polymers the observed $^1\text{O}_2$ phosphorescence signal exhibited long decay times with nonfirst-order kinetics at low temperatures. When matrix rigidity was decreased, either by a temperature increase or by changing the copolymer composition, the rise and decay rates of the observed $^1\text{O}_2$ signal increased, approaching those in liquid analogs. These changes in the $^1\text{O}_2$ phosphorescence signal were attributed to changes in the decay kinetics of the triplet state of the PS, since varying the aforementioned parameters modified the oxygen-PS encounter frequency. A mathematical approach consisting of a deconvolution of the triplet-state decay function from the observed $^1\text{O}_2$ phosphorescence signal enabled what the authors called the intrinsic $^1\text{O}_2$ lifetime in the polymer matrix to be obtained, in the same order as those in analogous liquids. Thus, $\tau_{\Delta,25^\circ\text{C}} \sim 38 \mu\text{s}$ for methyl propionate whereas $\tau_{\Delta,25^\circ\text{C}} \sim 20\text{--}25 \mu\text{s}$ for PMMA; for ethyl benzene $\tau_{\Delta,25^\circ\text{C}} \sim 26 \mu\text{s}$ whilst for polystyrene $\tau_{\Delta,25^\circ\text{C}} \sim 17\text{--}21 \mu\text{s}$. As can be seen, $^1\text{O}_2$ intrinsic lifetimes were slightly shorter than those in the liquid analogs. This was attributed to possible quenchers or deactivation processes in the polymer matrix, such as quenching by the ground-state PS or by sample impurities. Moreover, the increase in intrinsic $^1\text{O}_2$ lifetime when utilizing a perdeuterated polymer made the authors confident in affirming that nonradiative decay of $^1\text{O}_2$ in a polymer film was governed by the same parameters as in liquid solvents.

In a later study, Schiller and Müller⁴³ calculated $^1\text{O}_2$ lifetimes in various polymer matrices using the exponential correlation between the incremental rate constant (k_{xy}) of energy transfer from $^1\text{O}_2$ to terminal oscillators X-Y contained in solvent molecules and the energy (E_{xy}) of the highest fundamental vibration of the oscillator X-Y. They found that $^1\text{O}_2$ lifetime values in different polymers differed by less than in common solvents. This is likely due to the high proportion of the strongly deactivating C-H oscillators in most polymers. When they were containing O-H groups, the lifetime decreased substantially, although not as much as in H_2O . Molecular weight and density also influenced the deactivation, because these parameters modify the concentration of the molecular oscillators and thus, the value of the incremental deactivation constant (k_{xy}). The authors concluded that as a general statement, $^1\text{O}_2$ lifetimes in polymer films usually lay between 10 and 50 μs . Table 2.3 summarizes these values.

Table 2.3. $^1\text{O}_2$ lifetimes in polymer matrices, water and methyl propionate at 20 °C calculated by microcomputer program (literature data in parentheses). Data adapted from K. Schiller and F. W. Müller, *Polym. Int.*, 1991, 25, 19–22.

Matrix	Abbreviation	Composition	τ_{calc} (μs)
Water	H ₂ O		4.19 (4.2) ²⁷
Polyvinyl alcohol	PVAI		10.3
Polyvinyl formal	PVF	25 mol% OH	19.5
Polyvinyl ethylal	PVE	20 mol% OH	18.2
Polyvinyl ethylalbutyral	PVEB	PVE:PVB 1:1	18.1
Polyvinyl butyral	PVB	30 mol% OH	18.0
Polyvinyl chloride	PVC	56.8% Cl	28.1 (75.0)
Polyvinyl chloride (postchlor.)	PVC (n)	63.5% Cl	36.3
Polyvinylidene chloride	PVDC	73.2% Cl	37.5
Polyvinyl acetate	PVAc		31.4
Polyvinyl pyrrolidone	PVP		21.8
Cellulose hydrate	CH		12.9
Cellulose triacetate	CTA		43.0
Cellulose 2,5-acetate	CDA	17 mol% OH	36.2
Cellulose diacetate	CDA	33 mol% OH	30.4
Cellulose trinitrate	CTN		94.7
Cellulose dinitrate	CDN	33 mol% OH	41.0
Cellulose butyrate	CB		18.0
Cellulose acetobutyrate	CAB	Bu Ac:OH 24.1:74.5:1.4	18.1
Methyl cellulose	MeC	33 mol% OH	20.6
Ethyl cellulose	EC	17 mol% OH	24.3
Benzyl cellulose	BzC	33 mol% OH	22.7
Carboxymethyl cellulose	CMC	66 mol% COOH	21.2
Na ⁺ -carboxymethyl cellulose	CMCNa	66 mol% COO ⁻	43.1
Polyethylene terephthalate	PETP		33–7
Poly-(4,4'-isopropylidene-diphenylene-carbonate)	PC		22.9 (34.0)
Poly- <i>cis</i> -butadiene	PCB		15.45 (3.6)
Polystyrene	PS		18.9 (17–21, 25 °C) ⁴²
Polymethyl methacrylate	PMMA		25.8 (20–25, 25 °C) ⁴²
Methyl propionate	MeP		28.7 (38.0) ⁴⁴
Polytetrafluoroethylene (Nafion 510)	PTFE		4.0×10^4 (3.6×10^2) ⁴⁵

Some years later, Zebger *et al.*⁴⁶ reported a study in which $^1\text{O}_2$ images from films of polymeric blends with an immobilized PS were created. Indeed, $^1\text{O}_2$ phosphorescence was used as the optical probe to be detected to create the images. Additionally, they proposed a mathematical model to predict the changes in $^1\text{O}_2$ intensity in the phase boundaries.

Since this last report by Zebger *et al.*, literature examining the properties of $^1\text{O}_2$ generated within a polymer film or matrix has been very limited. The last report in this field is a recent work from Suchánek *et al.*,⁴⁷ describing the influence of temperature on the kinetics of $^1\text{O}_2$ photosensitized by an encapsulated or surface adsorbed porphyrin to polystyrene nanofibers. As in the work by Clough *et al.* (*vide supra*) these authors found that $^1\text{O}_2$ and triplet-state phosphorescence signals of the encapsulated PS were dependent on temperature, although in this case they were following first-order kinetics. Submerging these PS-encapsulating nanofibers in H₂O or D₂O afforded

similar values of the lifetime constants, revealing no marked influence of the external environment on the $^1\text{O}_2$ lifetime of this system. Conversely, when nanofibers with externally adsorbed TMPyP were submerged in H_2O , $^1\text{O}_2$ signal showed almost no dependence on temperature, consistent with a complete deactivation in the homogeneous aqueous medium.

In other experiments by Gao *et al.*⁴⁸ and Poulsen *et al.*,^{49,50} photosensitized- $^1\text{O}_2$ phosphorescence was employed as the optical probe to monitor oxygen sorption into a polymer and hence, determine its oxygen diffusion coefficient, D . With this technique, the authors have been able to further characterize oxygen diffusion in organic polymers that had not been previously investigated. For instance, $D_{25\text{ }^\circ\text{C}} = (2.3 \pm 0.3) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ for polystyrene,⁴⁸ or it was between ~ 2.2 and $5.8 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ for different samples of poly(ethylene-*co*-norbornene).⁴⁹

In summary, there has been a notable advance since the first indirect detection of $^1\text{O}_2$ in polymer films. Time-resolved phosphorescence studies have shown $^1\text{O}_2$ signals that do not follow first-order kinetics in some polymer matrices, which thereby need a more laborious mathematical treatment to acquire the intrinsic $^1\text{O}_2$ lifetime. It is not clear, though, why $^1\text{O}_2$ presents different apparent kinetics depending on the type of sample and PS examined, although it might be a consequence of the triplet decay kinetics or the frequency of the oxygen-triplet state encounter in a certain polymer. To our knowledge, no investigations in this direction have been reported, though. Computational methods have proved to be suitable for determining $^1\text{O}_2$ lifetime in a wide range of polymers, and $^1\text{O}_2$ itself has been used as a spectroscopic probe to quantify oxygen diffusion in polymers. Last experiments in this field indicate that $^1\text{O}_2$ properties in polymeric nanostructures may differ slightly from those of the traditional films.

2.5.3. Heterogeneous Nanoparticle-Based Environments

The study of $^1\text{O}_2$ properties in nanodevices has emerged as a new interdisciplinary research field since nanoparticles can be ideal carriers for PS molecules for PDT. Moreover, some nanomaterials, *e.g.* TiO_2 , ZnO , porous silicon and metal nanoparticles can generate $^1\text{O}_2$, thus exerting a phototherapeutic effect. Therefore, there are a growing number of reports available dealing with the production and deactivation of $^1\text{O}_2$ by different photosensitizing nanoplatforms.⁵¹ Herein, we revise some of the most notorious examples of $^1\text{O}_2$ deactivation inside nanoparticles, focusing our discussion in the values of τ_Δ and its diffusion distance.

2.5.3.1. Biodegradable Nanoparticles. Biodegradable nanoparticles (NPs) have been explored for more than one decade as drug-delivery vehicles due to their biocompatibility, their ability to encapsulate hydrophobic PSs and their controlled release. There exist several publications where NPs of different biodegradable materials are prepared incorporating different sorts

of PSs, including NPs of poly(lactic-co-glycolic acid) (PLGA), liposomes and human serum albumin (HSA) NPs.^{52,53} Nevertheless, only a few publications have gone into detail on investigating the mechanisms of $^1\text{O}_2$ formation and deactivation within these systems, comprising mainly HSA NPs and liposomes.

HSA NPs are promising systems because of their suitable biodegradability. Wacker *et al.* prepared HSA NPs loaded with two different hydrophobic PSs, 5,10,15,20-tetrakis(3-hydroxyphenyl)-chlorin (mTHPC) and 5,10,15,20-tetrakis(3-hydroxyphenyl)-porphyrin (mTHPP). Photosensitized $^1\text{O}_2$ by mTHPP- or mTHPC-loaded HSA NPs suspended in D_2O had a lifetime of $65 \pm 3 \mu\text{s}$ and $66 \pm 4 \mu\text{s}$, respectively, the same values than rose bengal-photosensitized $^1\text{O}_2$ in neat D_2O ($64 \pm 3 \mu\text{s}$), in good agreement with literature values.⁵⁴ This fact pointed out that $^1\text{O}_2$ is not quenched after its generation and it reaches the external aqueous environment. However, the $^1\text{O}_2$ quantum yield was dramatically reduced for both encapsulated PSs ($\Phi_{\Delta} = 0.03$ for both samples) compared to their quantum yield in ethanol solution ($\Phi_{\Delta} = 0.63$ and 0.65 for mTHPP and mTHPC, respectively). This reduction was attributed not only to PS interactions caused by the high local PS concentration but also to the low local oxygen concentration inside these systems.⁵³ In line with this, triplet excited states of the encapsulated PSs showed longer lifetime values than in homogeneous solution, which indicated an inefficient quenching process by molecular oxygen and further supported the model of a low local oxygen concentration inside the NPs.

The $^1\text{O}_2$ deactivation rate in heterogeneous systems depends not only on the environment where it is produced but also on the length over which it can diffuse, since the various environments encountered during its diffusion will also influence its deactivation. This has been discussed by Molnár *et al.*,⁵⁵ who studied $^1\text{O}_2$ and triplet-state kinetics of two different PSs, haematoporphyrin (HpD) and protoporphyrin IX (PpIX), encapsulated in L- α -phosphatidylcholine (PPC) liposomes. Time-resolved phosphorescence kinetics of $^1\text{O}_2$ at 1278 nm provided three time constants for both sorts of liposomes in aqueous suspension, two of which corresponded to the rate constants of triplet decay phosphorescence. This indicated that the PS was localized in two different areas of the lipid bilayer.^{55,56} On the contrary, $^1\text{O}_2$ decay was monoexponential, with a lifetime of $\sim 8 \mu\text{s}$ in the H_2O -based suspension for both PSs. Although a biexponential decay of $^1\text{O}_2$ was expected due to the presence of two independent PS-triplet species, the monoexponential decay revealed no apparent influence of the photosensitization site on the observed $^1\text{O}_2$ decay rate, which was an average between that in lipid phase ($12.2 \mu\text{s}$)⁵⁷ and in neat H_2O . Therefore, the kinetic model stated by Lee and Rodgers was valid in this case, denoting that $^1\text{O}_2$ could easily escape from inside the liposome and diffuse out to the aqueous medium during its lifetime.^{58,59} According to this model, many other researchers have found that time-resolved phosphorescence signals of photosensitized $^1\text{O}_2$ in liposomal aqueous suspensions yield a monoexponential decay with a lifetime of $\sim 3 \mu\text{s}$.^{56,60} This value corresponds to that of $^1\text{O}_2$ in H_2O ,²¹ which suggests that,

in these systems, $^1\text{O}_2$ would predominantly decay in the external aqueous medium. Considering that the diffusion constant of oxygen in dimyristoyl phosphatidylcholine (DMPC) liposomes at 21 °C is $1.5 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$,⁶¹ it emerges that $^1\text{O}_2$ would likely diffuse more than 100 nm in 3 times its lifetime (10 μs) (eqn (2.5)), which is a distance approximately 25-fold larger than the thickness of the lipid bilayer.⁵⁷ Hoebeke *et al.*,⁶² however, observed a slow diffusion rate of $^1\text{O}_2$ generated inside DMPC liposomal vesicles with bound merocyanine 540 (MC540). This was examined by using the isotopic effect of D_2O and the quencher effect of sodium azide. Taking into account different equations that consider the rate of $^1\text{O}_2$ in both environments, *i.e.* inside the bilayer and outside the aqueous medium, they concluded that $^1\text{O}_2$ spent more than 87% of its lifetime in the lipophilic environment of the bilayer.

Ehrenberg *et al.*⁵⁷ found $^1\text{O}_2$ decay times of 5 or 71 μs for DMPC liposomes in H_2O or D_2O , respectively. Furthermore, they achieved an estimation of the lifetime of $^1\text{O}_2$ in pure DMPC lipids, $\tau_{\Delta} \sim 36.4 \mu\text{s}$. A complementary study was carried out by Baier *et al.*⁵⁹ in pure PPC, which is a major cellular constituent. In order to obtain a system composed of pure PPC, the lipid was deposited into a glass plate incorporating Photofrin as a PS. Under these conditions, $^1\text{O}_2$ decay time was $14 \pm 2 \mu\text{s}$. Further experiments in living HT29 cells showed a $\tau_{\Delta} = 10 \pm 3 \mu\text{s}$, comparable to the decay time in pure PPC and being mainly attributed to $^1\text{O}_2$ decay inside the cells. However, the slight reduction in τ_{Δ} in the latter case was likely related to the cellular microenvironment where $^1\text{O}_2$ was diffusing and consequently, decaying. Hence, when liposomes are located in biological samples, the $^1\text{O}_2$ lifetime seems to significantly decrease due to additional deactivation by cellular quenchers. For instance, García-Díaz *et al.*⁶³ studied a folate-targeted liposomal formulation (POPC/OOPS/FA-PEG-DSPE, 90:10:0.1 molar ratio, FR-targeted liposomes) internalized in HeLa cells. The liposomes were entrapping the PS 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine zinc (ZnTPP), whereas after incubation HeLa cells were resuspended in D_2O -based PBS. This system provided a $\tau_{\Delta} = 1.5 \pm 0.4 \mu\text{s}$, which is much shorter than the typical value in D_2O (60–70 μs) or even shorter than the values observed in other liposomal suspensions (*vide supra*), indicating that $^1\text{O}_2$ may be substantially quenched in these cells.

Altogether, these results seem to indicate that, in most cases, τ_{Δ} is an average of the time spent by $^1\text{O}_2$ in the various phases of a microheterogeneous system. This is what Martínez *et al.*⁶⁴ illustrated in their work, determining $^1\text{O}_2$ decay rates in complex micellar and microemulsion systems by a simplified model of two pseudophases (aqueous and lipophilic). Fourteen years later, Hackbarth *et al.*⁶⁵ used small unilamellar vesicles (SUVs) for modeling the general behavior of heterogeneous samples. Their conclusions signaled, in agreement with aforementioned observations, that $^1\text{O}_2$ diffusion influences its deactivation kinetics. Nevertheless, they suggested that $^1\text{O}_2$ lifetime was not simply an average of the decay in the involved phases; it was likely dependent on the exact place of its generation within the liposome as well as on the radiative rate constants in the different phases. With these results, they afforded novel insights into $^1\text{O}_2$ deactivation mechanisms in microheterogeneous systems.

2.5.3.2. Porous Silica and Zeolite Nanoparticles. τ_{Δ} in porous silica NPs was early studied by Iu *et al.* using direct time-resolved phosphorescence detection at 1275 nm.⁶⁶ In their study two different PSs in benzene were used: pyrene, which was not adsorbed onto NPs and 2-acetonaphthone that was bound to silanol groups through H-bonds. τ_{Δ} of the composites was $26.2 \pm 0.5 \mu\text{s}$ for pyrene but $17.5 \pm 0.2 \mu\text{s}$ for the 2-acetonaphthone silica NPs. Moreover, for the 2-acetonaphthone silica NPs, they found that the narrower the pores were, the lower τ_{Δ} was (*i.e.* $\tau_{\Delta} = 14.4 \pm 0.2 \mu\text{s}$ for 60 Å pore and $17.5 \pm 0.2 \mu\text{s}$ for 150 Å pores). All these striking results were explained in terms of a nonradiative $^1\text{O}_2$ quenching by hydrogen-bonded water and silanol groups on the silica gel pores. They proposed that $^1\text{O}_2$ was generated inside the silica pores and then rebounded among the silica surfaces until it was eventually quenched. Moreover, as the collision frequency of $^1\text{O}_2$ is higher in narrow channels, the $^1\text{O}_2$ lifetime shortens.

Also in the field of silica NPs, quenching of $^1\text{O}_2$ has been recently detected in methylene blue-loaded amino- and mannose-targeted mesoporous silica nanoparticles (MSNPs) in ethanol.⁶⁷ In their study, the authors show that the lifetime is significantly shortened for the NP conjugates (*ca.* 4 μs) compared to the unbound PS in ethanol (*ca.* 15 μs) and propose three main contributions that may account for this observation: (a) quenching by free amino groups on the NP surface, (b) quenching by hydrogen-bonded water and silanol groups in the mesopores, and (c) enhancement of these processes by the increased wall-collision frequency in the narrow silica mesoporous channels.

Quenching of $^1\text{O}_2$ has also been reported for nanoporous alumina membranes functionalized with amino-silane agents that allowed the superficial binding of tetrakis(*p*-sulfonatophenyl)porphyrin (TPPS) through ionic interactions.⁶⁸ The τ_{Δ} for these conjugates was significantly reduced compared to τ_{Δ} of free TPPS in ethanol solution (9.9 μs), *i.e.* for the membranes with a pore diameter of 40 nm a lifetime of 3.7 μs was reported and for pores of 80 nm the lifetime was 6.4 μs . Taking into account previous results it was suggested that $^1\text{O}_2$ was likely generated inside the nanochannels and subsequently underwent serious collisional quenching dynamics with the silicate inner surface.

Apart from the pore size, the composition of the pores also affects τ_{Δ} . Using benzophenone as a PS, Jockusch *et al.* compared the τ_{Δ} in silica and in Y-zeolite $[\text{Na}_x(\text{AlO}_2)_y(\text{SiO}_2)_z]$ dry NPs.⁶⁹ Their findings revealed that τ_{Δ} varies with the alumina content of the nanomaterial, shortening from $64 \pm 2 \mu\text{s}$ for silica NPs to $35 \pm 0.1 \mu\text{s}$ for zeolites containing 20% of alumina and to $7.9 \pm 0.1 \mu\text{s}$ for zeolites containing 97.6% of alumina. They also proposed that in addition to the alumina anions, the presence of charge-balancing associated cations probably played an important role in the quenching of $^1\text{O}_2$.

Finally, the solvent where the NPs are dispersed plays an important role in $^1\text{O}_2$ deactivation. Spectroscopic and lifetime studies on zeolite catalysts in D_2O showed a monoexponential decay with a lifetime of 45.6 μs , suggesting

some quenching by either the zeolite or the water released through exchange processes.⁷⁰ On the contrary, suspensions in 1,1,1,2,3,4,4,5,5,5-decafluoropentane (DFP) systematically showed biexponential behavior with lifetimes around 470 and 7 μ s. These results led to the conclusion that a large fraction of $^1\text{O}_2$ exits the zeolite in DFP and is thus available for conventional oxidative processes outside the zeolite micropores.

2.5.4. Cellular Systems

2.5.4.1. Mammalian Cells. Studying $^1\text{O}_2$ properties in mammalian cells has been a well-pursued objective over more than twenty years. Technical difficulties, such as a lack of adequately sensitive detectors at NIR wavelengths, the low efficiency of $^1\text{O}_2$ phosphorescence in H_2O and the complexity of the cellular environment itself have rendered it difficult to ascertain which is the $^1\text{O}_2$ lifetime and its diffusion radius in a cell, and a strong debate exists among the scientists of this field.

The first attempts to assess $^1\text{O}_2$ lifetime in a cell were carried out independently by Moan and Berg³¹ and Baker and Kanofsky.⁷¹ These were actually indirect measurements based on the photodegradation rates of porphyrins, in the former case, or on experiments using lysed cells, in the latter case, which yielded $^1\text{O}_2$ lifetime values in the ranges between 10–40 ns and 170–320 ns, respectively. In view of these extremely short lifetimes compared to the value in neat H_2O (3.5 μ s, *vide supra*) and considering that the cell is a microheterogeneous milieu containing H_2O , organelles, and a vast number of macromolecules, it was long believed that $^1\text{O}_2$ readily reacted with these cellular components (quenchers) after generation, provoking this remarkable reduction in its lifetime. The first direct measurements of $^1\text{O}_2$ phosphorescence in living-cell suspensions provided lifetime values somewhat larger than the previous ones (*i.e.* 4–80 μ s).⁷² In 2002, though, the use of a novel photomultiplier tube for $^1\text{O}_2$ detection allowed Niedre *et al.* to further advance the detection of $^1\text{O}_2$ luminescence in cell suspensions previously incubated with ALS4Pc,⁷³ although apparently only 19% of the signal was coming from $^1\text{O}_2$ generated intracellularly. Their signals were fit with two components corresponding to $^1\text{O}_2$, one with $\tau_{\Delta} = 3.2 \pm 0.5 \mu$ s and the second with $\tau_{\Delta} = 0.6 \pm 0.4 \mu$ s, revealing the presence of $^1\text{O}_2$ decaying in H_2O in the case of the longer component, and $^1\text{O}_2$ decaying inside the cells in the case of the shorter component. Thus, their results pointed out once more that $^1\text{O}_2$ lifetime was importantly reduced inside a cell due to cellular quenchers.

A greater advance was made by Snyder *et al.* who constructed a custom-made NIR microscope⁷⁴ that served as a direct spatially resolved optical probe for $^1\text{O}_2$ detection at the single-cell level.⁷⁵ This detection system, within the constraints of the available spatial resolution, was advantageous in ensuring that the $^1\text{O}_2$ signal observed was coming mainly from inside the cell and not from the outside. In their studies,^{75–78} the authors denoted for the first time that $^1\text{O}_2$ lifetime inside a cell might be governed by solvent

effects instead of quenching by cellular components, estimating a $^1\text{O}_2$ lifetime of $\sim 2.7 \mu\text{s}$ inside a cell. Furthermore, assuming the diffusion coefficient of O_2 in a cell to be the same as that in H_2O ($2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$) and applying eqn (2.5), they made the first estimation of the distance traveled by $^1\text{O}_2$ in a cell: around 270 nm, the dimension of an organelle, in a time twice its lifetime. These authors also established that addition of 0.75 mM BSA, a $^1\text{O}_2$ quencher that does not enter the cell during the time course of these experiments,⁷⁹ was rather convenient to detect only $^1\text{O}_2$ coming from inside the cell.

On the other hand, Jiménez-Banzo *et al.* reported a study comparing two PSs, TMPyP and TPPS, which localize in two different cellular compartments (nucleus and lysosomes, respectively). At the same time they compared their results in D_2O - and H_2O -based cellular suspensions.⁸⁰ They found that $^1\text{O}_2$ sensitized in the nucleus had a $\tau_{\Delta} \sim 24 \mu\text{s}$ for D_2O -based cellular suspensions whereas it was $\sim 1.7 \mu\text{s}$ for H_2O -based cellular suspensions. Hence, their results were conveying the two previous trends: on the one hand, there was a remarkable difference in τ_{Δ} when H_2O was replaced by D_2O according to the isotope effects, meaning that one of the main pathways for $^1\text{O}_2$ deactivation was electronic to vibrational energy transfer due to the solvent; on the other hand, the value of $\sim 1.7 \mu\text{s}$ for H_2O -based cellular suspensions was clearly less than that in neat H_2O , suggesting that $^1\text{O}_2$ deactivation by cellular components was also occurring. Indeed, addition of 0.75 mM BSA in the case of D_2O -based cellular suspensions reduced τ_{Δ} to 6 μs , confirming that $^1\text{O}_2$ was diffusing out of the cell and that the quenching by cellular components, expressed as $k_{\text{q}}[\text{Q}] \sim 2.5 \times 10^{-5} \text{ s}^{-1}$, was larger than that estimated previously.⁷⁵⁻⁷⁸ More importantly, there were substantial differences when $^1\text{O}_2$ was generated in lysosomes (TPPS incubation): its lifetime was $14 \pm 2 \mu\text{s}$ and $1.5 \pm 1 \mu\text{s}$ in D_2O - and H_2O -based cellular suspensions, respectively, thus significantly shorter than for $^1\text{O}_2$ generated in the nucleus, and the addition of 0.77 mM BSA produced no significant $^1\text{O}_2$ quenching, indicating that $^1\text{O}_2$ was not able to diffuse out of the cell when photosensitized in lysosomes. With these values and on the assumption that the oxygen diffusion coefficient should be the same as in H_2O , they estimated a diffusion distance of $^1\text{O}_2$ up to $\sim 0.4 \mu\text{m}$ when sensitized in the nucleus or up to $0.9 \mu\text{m}$ when sensitized in the lysosomes in D_2O -based cells before complete deactivation ($t = 5\tau_{\Delta}$). Altogether, these results seemed to indicate that the actual lifetime of $^1\text{O}_2$ within a cell may be $13 \pm 2 \mu\text{s}$, differing from that observed for TMPyP since the actual lifetime of $^1\text{O}_2$ within a cell may be an average of that at the various cell compartments through which it diffuses. Additionally, $^1\text{O}_2$ lifetime in cells seemed to strongly depend on the organelle where it was generated (Figure 2.5).

A more recent advance, made independently by Kuimova *et al.*,⁸¹ Schlothauer *et al.*⁸² and. Hackbarth *et al.*,⁸³ has been the observation that $^1\text{O}_2$ phosphorescence kinetics was evolving with the elapsed irradiation time. Data from Kuimova *et al.* clearly showed an irradiation-dependent decrease in both the rate of $^1\text{O}_2$ formation and decay. This decrease was attributed to an increase in intracellular viscosity during PDT, since viscosity influences bimolecular processes such as $^1\text{O}_2$ formation from the triplet state and $^1\text{O}_2$

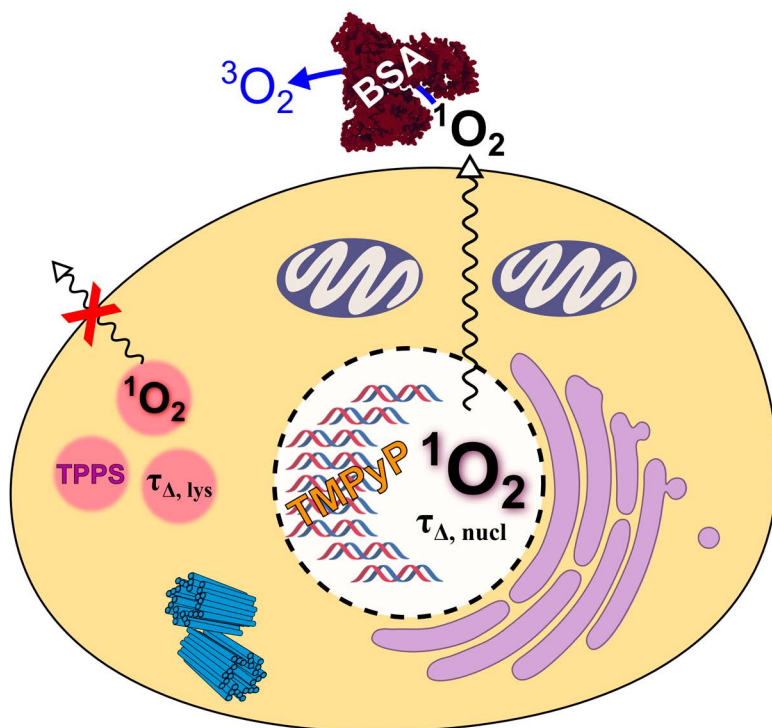


Figure 2.5. Schematic illustration of the various outcomes of $^1\text{O}_2$ when generated in different organelles in the cell.

decay by collisions with intracellular quenchers.^{81,84,85} On the other hand, Hackbarth *et al.* detected that the $^1\text{O}_2$ phosphorescence signal exhibited two components, one belonging to $^1\text{O}_2$ from inside the lipid bilayer and the other belonging to $^1\text{O}_2$ found in the aqueous medium. This highlighted that in cellular systems it might not be valid to assume that $^1\text{O}_2$ observed decay is an average between the decay of the various compartments, as stated previously for other heterogeneous systems (eqn (2.7)).⁴¹ Furthermore, they attributed the changes over time of the $^1\text{O}_2$ signal to diffusion and localization of $^1\text{O}_2$ in different subcellular compartments. More recent experiments⁷⁹ introduced some doubts over the above interpretations, though. Indeed, it was demonstrated that the addition of the extracellular quencher BSA prevented the observed changes in τ_{Δ} and τ_T upon a prolonged irradiation time. This means that the evolution of the $^1\text{O}_2$ signal was due to an increase in the amount of hydrophilic PS leaking from the cells to the extracellular medium. Conversely, in the same study the authors found that for pheophorbide a, a hydrophobic PS, there was indeed an evolution of the $^1\text{O}_2$ signal, even in the presence of BSA. This surprising result reinforced the idea that changes in viscosity during PDT might play a role in $^1\text{O}_2$ kinetics inside the cell. In line with these observed differences depending on the physical properties of the

PS and thus, on its subcellular localization, it was found that the quenching rates of $^1\text{O}_2$ by NaN_3 depended as well on the PS localization, *i.e.* they were influenced by the local environment where $^1\text{O}_2$ was produced.^{32,79}

In parallel studies to further elucidate $^1\text{O}_2$ behavior in a cell, Hatz *et al.*³² have started to estimate the actual intracellular diffusion coefficient of oxygen, D . The reason for this is based on independent studies reporting that diffusion coefficients of small solutes in intracellular domains, including O_2 , are smaller than those in neat H_2O , which is attributed, among other factors, to local regions of high viscosity in the cell.^{86–88} By comparing results of bulk sucrose solutions (which serve to increase the viscosity of the solution and decrease the dissolved oxygen concentration) with single-cell data they made a first estimation of an apparent oxygen diffusion coefficient in subcellular domains, which pointed out to be $D \sim 2 \times 10^{-6} - 4 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, *i.e.* around 5–10 times smaller than that in H_2O . This means that supposing an intracellular $^1\text{O}_2$ lifetime of 3 μs , the apparent diffusion of $^1\text{O}_2$ would have a radius of around 100 nm.

In light of all these controversial results it is clear that new or refined techniques are in high demand to deepen the understanding of intracellular $^1\text{O}_2$. An example of such is the new NIR-sensitive 2D-array InGaAs detector (NIRvana) coupled to an imaging spectrograph that allows acquiring spectral images where one dimension is spatial and the other, spectral.⁸⁹ This system, although still in a preliminary stage, seems to be promising since the signal-to-noise ratio is somewhat larger and acquisition time of $^1\text{O}_2$ -based images can considerably be reduced (only 5 s are needed). Furthermore, it has been proved useful to perform a real-time imaging of the NIR phosphorescence of $^1\text{O}_2$ and the PS simultaneously with the visible fluorescence of the PS, while it would also enable to distinguish and separate $^1\text{O}_2$ phosphorescence from the NIR luminescence of the PS.

2.5.4.2. Prokaryotic Cells. Unlike for eukaryotic cells, the amount of published literature on the deactivation mechanisms and properties of $^1\text{O}_2$ in prokaryotic cells is considerably lower, with investigations leading to unequivocal understanding about the site of generation and decay of $^1\text{O}_2$ in this type of cells.

The first study was reported by Maisch *et al.*, who studied the decay of $^1\text{O}_2$ luminescence signals in suspensions of the gram-positive model bacteria *S. aureus* incubated with Photofrin.⁹⁰ They observed a $^1\text{O}_2$ decay time of 6 μs , which is longer than that in H_2O but shorter than that reported in PPC (14 μs).⁵⁹ The fact that the value of $^1\text{O}_2$ decay is closer to that in H_2O suggests that there is a strong influence of the solvent on quenching this species. This means that $^1\text{O}_2$ molecules are able to escape to the external aqueous media. This is reasonable with the hypothesis that $^1\text{O}_2$ might be generated in the membranes of bacteria given that the membrane thickness of *S. aureus* is 0.2 μm and that the range of the diffusion length of $^1\text{O}_2$ in cellular membranes is around 0.3 μm .⁵⁹

$^1\text{O}_2$ decay kinetics in suspensions of the model Gram-negative bacteria *E. coli* incubated with the cationic TMPyP were reported by Ragàs *et al.*⁹¹ Spectroscopic

measurements of internalized TMPyP revealed that this PS has a dual localization in *E. coli*: a fraction of TMPyP is bound to the cell wall, whereas the other is internalized and bound to cytosolic nucleic acids. Moreover, both populations were able to produce $^1\text{O}_2$. In PBS-D₂O-based suspensions, signals rose with $\tau_{\text{T1}} = 2.5 \pm 1 \mu\text{s}$ and $\tau_{\text{T2}} = 20 \pm 2 \mu\text{s}$, and decayed with $\tau_{\Delta} = 66 \pm 4 \mu\text{s}$, whereas in PBS-H₂O-based suspensions there was a monoexponential rise $\tau_{\text{T1}} = 2.5 \pm 1 \mu\text{s}$ and a biexponential decay comprising $\tau_{\Delta} = 3.5 \pm 1 \mu\text{s}$ and $\tau_{\text{T2}} = 20 \pm 2 \mu\text{s}$. Thus, $^1\text{O}_2$ decayed with a single lifetime irrespective of the site of formation, suggesting that in these cells there was indeed a fast equilibration between internal and external populations of $^1\text{O}_2$ before its decay. Furthermore, τ_{Δ} values were similar to those observed in aqueous solution, meaning that $^1\text{O}_2$ was able to cross the cell wall and reach the external aqueous medium.

In a subsequent study,⁹² variations in $^1\text{O}_2$ kinetics depending on the incubated PS in *E. coli* suspensions were determined. Three cationic PSs belonging to three different families were tested: new methylene blue (NMB), a phenothiazine, zinc(II) tetramethyltetrapyrro[3,4-b:39,49-g:30,40-1:3-4-*q*] porphyrinium salt (ZnTMPyPz), a porphyrine, and ACS268, a hydrophobic porphyrin with a cationic alkyl chain. A clear dependency on the type of PS, *i.e.* its localization, was observed for the kinetics of production and decay of $^1\text{O}_2$ phosphorescence signal. Briefly, NMB and ZnTMPyP were located in the external structure of the cell wall, yet the outcome of $^1\text{O}_2$ was dissimilar due to their different hydrophilicity. NMB, as a water soluble PS, was able to produce $^1\text{O}_2$ that was decaying with the typical lifetime values in these neat solvents, indicating it was diffusing out of the cell. Nevertheless, ZnTMPyP tends to become aggregated in aqueous medium, thus not producing $^1\text{O}_2$. On the contrary, ACS268-photosensitized $^1\text{O}_2$ decayed with a lifetime of $2.1 \pm 1 \mu\text{s}$ in PBS and $5.2 \pm 1 \mu\text{s}$ in D₂O-based PBS, both lifetimes significantly shorter than in their respective neat solvents. This would point to a faster deactivation due to cellular components and consequently, to a deeper position of this PS into the cell wall. On the other hand, the aforementioned data revealed as well that $^1\text{O}_2$ underwent a fast equilibration before its decay in this microheterogeneous system.⁴¹ Assuming $k_{\text{eq}} \approx 1$ and $f_{\text{m}} \approx 0.0012$ for their suspensions in $5 \times 10^8 \text{ CFU mL}^{-1}$, τ_{Δ} was estimated to be $\sim 7 \text{ ns}$ in a bacterial cell (eqn (2.7)).

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Section II

Production of Singlet Oxygen

Chapter 3

Water-Soluble Carriers of Singlet Oxygen for Biological Media

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3.1. Introduction

Many biochemical and photochemical processes generate singlet oxygen ($^1\text{O}_2$, $^1\Delta_g$) *in vivo*. In particular, it is known that this species is formed beside other reactive oxygen species (ROS) such as $\text{O}_2^{\cdot-}$, $\text{OH}^{\cdot}$ or H_2O_2 during the so-called “photodynamic effect” that involves the simultaneous effect of oxygen, a photosensitizer and visible or UV light on a biological target. It is generally admitted that $^1\text{O}_2$ is the main active species in this process. However, considering the complexity of biological systems and the great variety of active species generated by photochemistry, it is difficult to assess clearly the role of each species in the resulting biological effects. Thus, researchers have sought methods to generate $^1\text{O}_2$ free of other ROS in biological media. The direct excitation of $^3\text{O}_2$ with IR radiation at 1270 nm specifically generates $^1\text{O}_2$ without requiring any photosensitizer likely to induce side reactions.³ However, this apparently simple method necessitates a powerful IR laser specially designed to emit at this particular wavelength and the process is very inefficient since the electronic transition from $^3\text{O}_2$ to $^1\text{O}_2$ is highly spin forbidden. A simpler alternative relies on the chemical generation of $^1\text{O}_2$ “in the dark” to avoid photochemical side reactions. Many chemical sources of singlet oxygen are available⁴ but the milder one is based on the thermolysis of naphthalene endoperoxides NO_2 (Figure 3.1). Indeed, following the seminal idea of Dufraisse (see below), several authors have exploited the phenomenon of reversible binding of oxygen to polycyclic aromatics to design water-soluble naphthalene derivatives able to behave as genuine carriers of $^1\text{O}_2$ suitable for biological media.

Several reviews have been devoted to the reversible [4+2] cycloaddition of $^1\text{O}_2$ on aromatic compounds^{5,6} and to the reactions of $^1\text{O}_2$ with biological targets.^{1,7} However, only short reviews are dedicated to water-soluble carriers of $^1\text{O}_2$ ⁸ and their applications in biochemistry and biology.² This chapter is focused more specifically on organic synthetic strategies used to design effective $^1\text{O}_2$ carriers and on the applications of water-soluble $^1\text{O}_2$ carriers to establish precisely the reactivity of $^1\text{O}_2$ with molecular, macromolecular or microbial targets of biological interest.

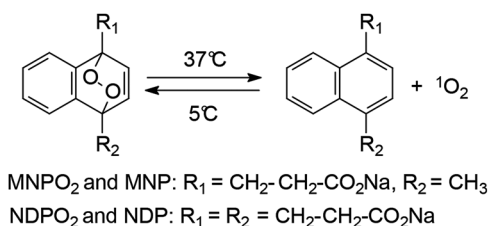


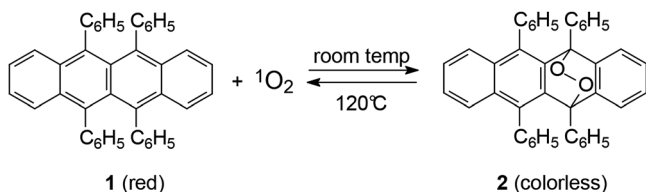
Figure 3.1. Mild chemical sources of singlet oxygen based on the thermolysis of naphthalene endoperoxides NO_2 .

3.2. Reversible Binding of Oxygen to Aromatic Compounds

3.2.1. Historical Background

As a rule of thumb, the reaction of oxygen with an organic molecule results in an irreversible degradation of the starting compound. However, in 1926, Moureu and Dufraisse made the astonishing discovery that rubrene **1** is able to bind covalently and reversibly oxygen (Figure 3.2).^{9,10}

Actually, when shaken with air in moderate sunlight, this ruby-red compound has the remarkable property of fixing a molecule of oxygen to form a colorless cyclic endoperoxide **2**. When heating this product to about 120 °C, it dissociates and releases almost all its oxygen in the free state while the original hydrocarbon is regenerated. Dufraisse named this phenomenon, which bears some resemblance to the reversible oxygenation of haemoglobin to oxyhaemoglobin, "*labile union of oxygen to carbon*". He subsequently showed that many anthracene derivatives yield, in the same way, thermodissociable endoperoxides. As he was a pharmacist, he thought immediately of the potential biological applications of this phenomenon. So, he devoted much effort to prepare aromatic endoperoxides being simultaneously water-soluble and dissociable at low temperature compatible with biological systems. One of the most significant results of his quest was achieved in 1942 when he showed that the 1,4-endoperoxide of 9,10-diphenyl-1,4-dimethoxyanthracene **3** (Figure 3.3) dissociated at room temperature by releasing an "activated" form of oxygen that was able to diffuse through a sheet of black paper and to impress a photographic plate.¹¹



In 1967, Wasserman¹² proved that a part of the oxygen released by the dissociation of aromatic endoperoxides was in a singlet excited state. Indeed, it showed that the endoperoxide of 9,10-diphenylanthracene **4** heated to 80 °C oxidized various substrates by giving the same products as those obtained with $^1\text{O}_2$ generated photochemically. This formation of $^1\text{O}_2$ through thermolysis of polycyclic aromatic endoperoxides is quite general since it was demonstrated during the decomposition of endoperoxides of many naphthalene, anthracene and tetracene derivatives.¹³ However, neither of the endoperoxides **3** and **4** are suitable models to design $^1\text{O}_2$ carriers for biological media since **3** is not stable in aqueous media because of its acetal structure and **4** dissociation is too slow at 37 °C. On the contrary, endoperoxides of 1,4-dialkyl naphthalenes NO_2 are stable in aqueous medium and they dissociate at moderate temperature.¹⁴ Dufraisse's dream finally became reality. Following this idea, Saito¹⁵ prepared in 1981 the first water-soluble carrier of $^1\text{O}_2$ by grafting on a naphthalene ring, a sodium propyl carboxylate and a methyl groups in the 1 and 4 positions respectively (**MNP**, see Figure 3.1). The corresponding endoperoxide **MNPO**₂ is actually able to release pure $^1\text{O}_2$ in aqueous medium but it exhibits a poor water solubility (10^{-2} M at pH 7.5) since it has only one hydrophilic group. To overcome this problem, Aubry and coworkers prepared in 1985 a similar, but much more water-soluble (>1 M) derivative, **NDP**, by grafting two sodium propylcarboxylate groups in the 1 and 4 positions of the naphthalene ring.^{16,17} Since then, the corresponding endoperoxide **NDPO**₂ has been used as a standard by biochemists and biologists to generate well-defined amounts of pure $^1\text{O}_2$ in aqueous media.

3.2.2. Reaction of $^1\text{O}_2$ with Aromatic Compounds

3.2.2.1. Structural Effects. Singlet oxygen ($^1\text{O}_2$, $^1\Delta_g$) may react according to a [4+2] cycloaddition with electron-rich aromatic substrates such as anthracene and higher members of the acene series. In contrast, unsubstituted benzene and naphthalene fail to react with $^1\text{O}_2$. The reactivity of aromatic hydrocarbons increases with the electron density of the substrate in agreement with the electrophilic nature of $^1\text{O}_2$. The comparison of anthracene, tetracene, and pentacene shows that the reactivity increases by about 2 orders of magnitude for each supplementary fused ring. The grafting of electron-releasing groups on the site of $^1\text{O}_2$ addition increases the rate constants in the order $\text{H} < \text{C}_6\text{H}_5 < \text{CH}_3 < \text{OCH}_3 < \text{N}(\text{CH}_3)_2$. For instance, 1-methyl-naphthalene slowly reacts with $^1\text{O}_2$, whereas naphthalene itself is completely unreactive. Steric strain is also an important parameter that can modify both the reactivity of the substrate and the regioselectivity of the cycloaddition of $^1\text{O}_2$. Peri-interactions between two neighboring methyl groups bound to a polycyclic aromatic hydrocarbon enhance its reactivity toward $^1\text{O}_2$ because the steric strain is somewhat relieved in the transition state. This phenomenon (Figure 3.4) explains why 1,8-dimethylnaphthalene **5** is 4 times more reactive than the 1,5-isomer **6**.¹⁸

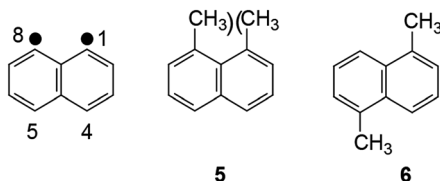


Figure 3.4. Explanation of the reason why 1,8-dimethylnaphthalene **5** is 4 times more reactive than the 1,5-isomer **6** towards singlet oxygen.

3.2.2.2. Solvent Effects. The rate constants of [4+2] cycloaddition of $^1\text{O}_2$ on organic substrates are generally considered as practically solvent independent, in agreement with a mechanism analogous to the “normal” Diels–Alder reaction. However, a detailed study comprising a wide range of solvents has revealed that the widely accepted dogma of solvent independency is wrong when the reaction is conducted in highly structured solvents such as *N*-methylformamide, formamide, or water.¹⁹ In particular, it was found that the rate constant of $^1\text{O}_2$ addition on NDP increases by about 2 orders of magnitude from methanol to water. This huge solvent effect has important practical consequences for the preparation of water-soluble endoperoxides by using a chemical²⁰ or a photochemical “primary” source of $^1\text{O}_2$. Indeed, the rate of peroxidation of naphthalenic carriers (see eqn (3.1) from right to left) is much faster in water than in an organic solvent in spite of the shorter $^1\text{O}_2$ lifetime in water.²¹

3.2.3. Cycloreversion of Endoperoxides Through Thermolysis

Two primary pathways of transformation may compete during the thermolysis of aromatic endoperoxides: cycloreversion, leading to parent substrate and oxygen, in a singlet or a triplet state; and homolytic cleavage of the peroxidic bond, followed by rearrangement to more or less stable diepoxides or by decomposition, leading to hydroxy-ketones or quinones. The ratio between cycloreversion and cleavage may be rationalized from the values of the relative activation energies. It appears that the activation enthalpy $\Delta H^\ddagger$ for cycloreversion increases from benzenic, to naphthalenic, to 1,4-anthracenic and then to 9,10-anthracenic endoperoxides. Consequently, cleavage may compete with cycloreversion only for 9,10-anthracenic endoperoxides and more condensed analogs.²² Therefore, water-soluble naphthalenic endoperoxides do not suffer from any cleavage upon heating and release all their oxygen as a mixture of $^1\text{O}_2$ and $^3\text{O}_2$.

3.3. Water-Soluble Naphthalenic Endoperoxides NO_2 as Singlet Oxygen Carriers

3.3.1. Design of Water-Soluble Carriers of $^1\text{O}_2$

Compared to the endoperoxide of 9,10-diphenylanthracene, the endoperoxide of 1,4-dimethylnaphthalene (**DMNO₂**) releases $^1\text{O}_2$ at lower temperature (37 °C *versus* 80 °C) and with a higher yield (76% *versus* 32%). **DMNO₂** is

almost the sole endoperoxide used to generate $^1\text{O}_2$ in organic solvents. Indeed, its precursor, 1,4-dimethylnaphthalene, is commercially available, fairly reactive towards $^1\text{O}_2$ and the corresponding endoperoxide is chemically stable and releases $^1\text{O}_2$ free of other reactive oxygen species, under mild conditions ($t_{1/2} \approx 5$ h at 25 °C).²³ Some polymers derived from 1,4-dimethylnaphthalene^{24–26} or 1-methylnaphthalene²⁷ have also been reported. The corresponding endoperoxides compounds have the advantage of being isolated from the reaction medium at the end of reaction by filtration and are also able to release $^1\text{O}_2$ at 37 °C. Poly(1,4-dimethyl-6-vinylnaphthalene)-1,4-endoperoxide²⁶ has shown some inactivation of enveloped viruses. However, because of their high hydrophobicity, such polymers mainly find applications in organic media. However, water-soluble and nontoxic derivatives of **DMN** can also act as efficient carriers of $^1\text{O}_2$ as they trap this species at low temperature (0–5 °C). The corresponding endoperoxides can be stored at –80 °C for months and release definite amounts of $^1\text{O}_2$ on warming at 37 °C.

3.3.2. Strategies for the Synthesis of Water-Soluble Carriers

Hydrophilic substituents grafted onto the naphthalene backbone must be insensitive to $^1\text{O}_2$ and to the photosensitizer or the chemical source of $^1\text{O}_2$ that are required to prepare the endoperoxide **NO₂**. Moreover, the hydrophilic function must not act as a $^1\text{O}_2$ physical quencher such as amino or phenol groups. Carboxylate, amide, phosphate, sulfonate, sulfate, sulfonamide, quaternary ammonium, alcohol and ether functions meet these requirements. Naphthalene itself does not react with $^1\text{O}_2$. The direct binding of one of the above electron-withdrawing groups to the aromatic core decreases its reactivity further. Therefore, at least one, and preferably two electron-donating groups must be present at the 1,4 positions to allow the [4+2] cycloaddition of $^1\text{O}_2$ and to stabilize the endoperoxide. Thus, the relevant starting structure is **DMN** to which water-solubilizing groups must be grafted. Figure 3.5 summarizes the possibilities of anchoring hydrophilic substituents on 1,4-dimethylnaphthalene, **DMN**.

From a synthetic viewpoint, substitution of the hydrogen at position 7 is not easy. On the other hand, introduction of an electron-withdrawing

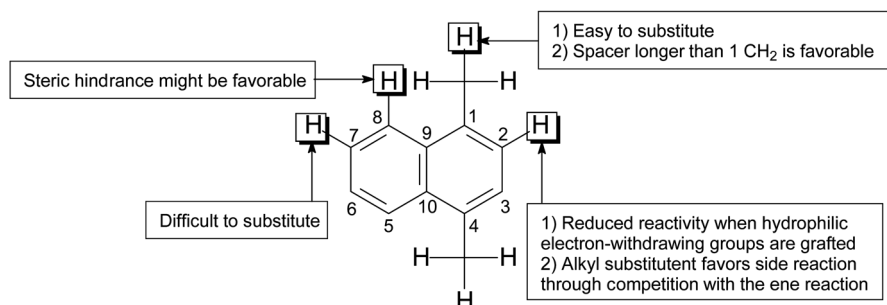


Figure 3.5. Possibilities for **DMN** modification by grafting hydrophilic groups.

hydrophilic group at position 2 would decrease the reactivity of the molecule towards $^1\text{O}_2$. Moreover, it has been observed that an alkyl group (e.g. methyl) can provide secondary products resulting from the ene reaction. The best position to introduce a hydrophilic substituent is thus on the methyl group at position 1. Indeed, one hydrogen atom can be easily substituted but a sufficiently long alkyl spacer bearing at least two CH_2 groups must separate the hydrophilic group from the 1,4 carbons of the naphthalene moiety.

The first water-soluble $^1\text{O}_2$ carriers reported by Saito¹⁵ and Aubry,^{16,17} i.e. **MNP** and **NDP**, were designed to possess the required properties. These compounds bear one or two sodium propanoate substituents, respectively, grafted on the 1,4 positions of the naphthalene core. The corresponding endoperoxides have been used as chemical sources of $^1\text{O}_2$ to assess the activity of $^1\text{O}_2$ towards chemical, biochemical or biological targets. However, these anionic compounds release $^1\text{O}_2$ anywhere in the aqueous phase, potentially far from the target. Subsequently, a second generation of carriers has been designed. They bear specific groups such as quaternary ammonium in the case of **MNEA** or nonionic hydrophilic groups for **NDMOL**, **DHPN**^{28,29} (see Figure 3.6) in order to confer a particular affinity for polynucleotides, negatively charged sites or intracellular targets to these molecules.

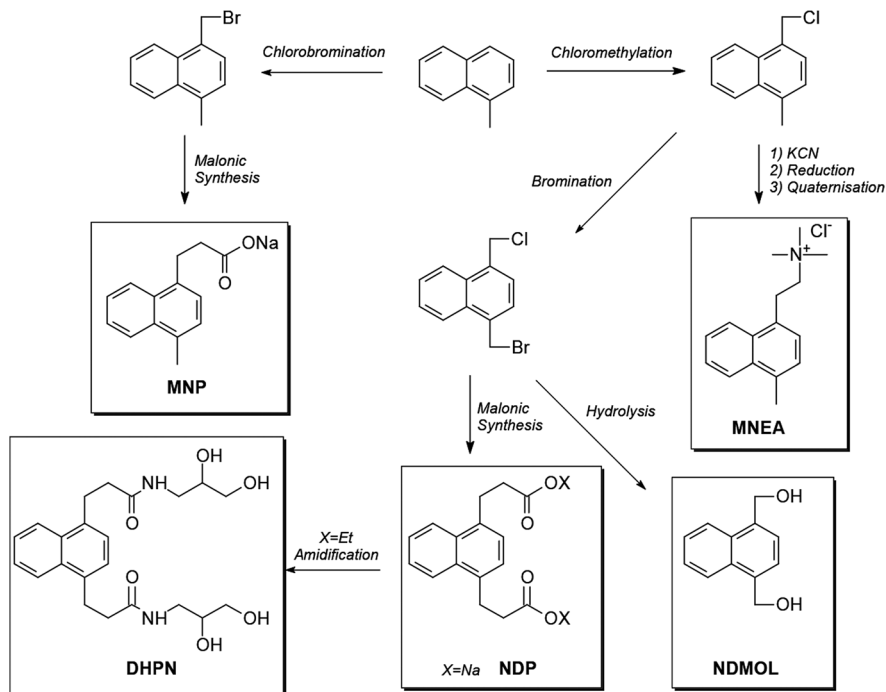


Figure 3.6. Synthesis of 1,4-disubstituted naphthalenic water-soluble carriers of $^1\text{O}_2$.

All the naphthalenic carriers **MNP**, **MNEA**, **NDMOL**, **NDP**, **MNEA** of $^1\text{O}_2$ used in biological media can be prepared from the (inexpensive) 1-methylnaphthalene using the general scheme presented in Figure 3.3.^{30,31} In the first step, 1-methylnaphthalene is chloro- or bromomethylated leading to sodium 4-methyl-1-naphthalenepropanoate **MNP**¹⁵ and 4-methyl-*N,N,N*-trimethyl-1-naphthalene ethanaminium chloride **MNEA**²⁸ bearing only one anionic or cationic solubilizing group respectively. The conventional route to bifunctional naphthalene derivatives, *i.e.* **NDMOL**, **NDP** and **DHPN**, starts with a double bromination of the (costly) **DMN**.^{30,32} An alternative access to 1,4-dihalogenomethylnaphthalene consists in the monobromination of 1-chloromethyl-4-methylnaphthalene which is then converted into the nonionic **NDMOL** or the anionic disodium 1,4-naphthalenedipropionate **NDP** according to previously reported methods.^{28,33} Synthesis of nonionic carrier *N,N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropylamide **DHPN**, involves amidification of the diethylester of **NDP**.⁸ All five carriers **MNP**, **MNEA**, **NDMOL**, **NDP**, **DHPN** exhibit sufficient water solubility (10^{-2} M) for most biological applications (see Table 3.1).

Besides **MNP**, **MNEA**, **NDP**, **NDMOL** and **DHPN**, other $^1\text{O}_2$ carriers based on water-soluble naphthalene derivatives have been designed as summarized in Tables 3.2 and 3.3.

3.3.3. Singlet Oxygenation of Naphthalenic Carriers

Two primary sources of $^1\text{O}_2$ can be used to prepare endoperoxides on a preparative scale: the regular photochemical method³⁹ (see eqn (3.1)) and the molybdate-catalyzed disproportionation of hydrogen peroxide²⁰ (see eqn (3.2)).

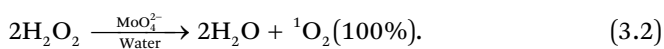
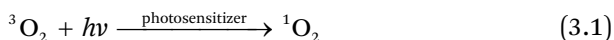


Table 3.1. Main physicochemical properties of $^1\text{O}_2$ carriers **MNP**, **MNEA**, **NDMOL**, **NDP** and **DHPN**.

Starting naphthalenes	MNP	MNEA	NDMOL	NDP	DHPN
Water solubility (M) ^a	10^{-2}	>0.1	0.95×10^{-2}	>1	
$10^6 (k_r + k_q) (\text{M}^{-1} \text{s}^{-1})^b$	7.0	1.4	0.4	2.8	1.0
Endoperoxides	MNPO₂	MNEAO₂	NDMOLO₂	NDPO₂	DHPNO₂
$t_{50\%}$ (min) ^c	23	23	70	23	22
$t_{95\%}$ (min) ^d	99	99	300	99	99
$^1\text{O}_2$ yield (%) ^e	45	65	51	50	59

^aIn H_2O at 20 °C.

^bOverall quenching rate constants of $^1\text{O}_2$ determined by flash photolysis in D_2O .

^cHalf-time of decomposition.

^dTime necessary to decompose 95% of naphthalenic endoperoxides.

^eCumulative yields of $^1\text{O}_2$ produced by thermolysis.

Table 3.2. Hydrophilic groups borne by water-soluble naphthalenic carriers of $^1\text{O}_2$.

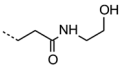
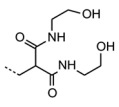
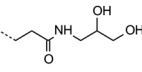
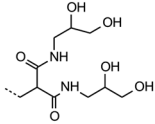
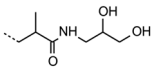
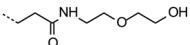
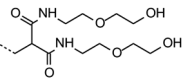
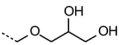
Ionic groups	
	a
	b
	g
Nonionic groups	
	c
	h
	d
	i
	e
	f
	j
	k
	l
	m
	n

Table 3.3. Chemical structures of water-soluble naphthalenic carriers of $^1\text{O}_2$. Overall rate constant ($k_r + k_q$) determined by flash photolysis in D_2O . Half-time for the thermal cleavage of endoperoxides ($t_{1/2}$) at 37 °C. *nd* is not determined.

No.	Abbreviation	R_1	R_2	R_3	R_4	$(k_r + k_q)/10^6 \text{ M}^{-1} \text{ s}^{-1}$	$t_{1/2}$ at 37 °C (min)	Ref.
7	MNEA	a	H	H	CH ₃	1.4	23	6
	MNP	b	H	H	CH ₃	7.0	23	15
	NDP	b	H	H	b	2.8	23	16
	DHPN	c	H	H	c	1.2	<i>nd</i>	34
		d	H	H	d	1.0	22	35
8		e	H	H	e	0.9	<i>nd</i>	34
9		f	H	H	f	<i>nd</i>	22	34
10		g	H	H	g	1.4	<i>nd</i>	34
11		h	H	H	h	0.1	<i>nd</i>	34
12		i	H	H	i	0.4	<i>nd</i>	34
13	NDMOL	j	H	H	j	0.2	<i>nd</i>	34
14		k	H	H	k	0.4	70	6
		l	H	H	l	0.1	<i>nd</i>	34
15	DHPON	m	H	H	m	197	<i>nd</i>	36
		CH ₃	b	H	b	13	319	34
		CH ₃	c	H	c	<i>nd</i>	441	34
		CH ₃	d	H	d	7.8	542	34
		CH ₃	h	H	h	<i>nd</i>	284	34
19		CH ₃	i	H	i	<i>nd</i>	582	34
20		b	CH ₃	CH ₃	b	22	63	34
21		b	H	b	H	13	318	34
	BNPE	<i>n</i> -Bu	H	H	b	<i>nd</i>	20	37
	DMNOH	CH ₃	n	H	CH ₃	<i>nd</i>	262	38

In both cases, the oxidation proceeds more rapidly in deuterated solvents (D_2O and CD_3OD) since the lifetime of $^1O_2$ is much longer in these solvents than in the protonated ones (55 and 210 μs *versus* 3 and 10 μs , respectively). The choice of the oxidizing method depends on the physicochemical properties of the naphthalenic compounds (water solubility, nature of the hydrophilic functions and reactivity towards $^1O_2$). For instance, the chemical process is ideal for peroxidizing salts of carboxylic naphthalene derivatives (**MNP** and **NDP**) because the endoperoxides can be recovered readily by precipitating the acidic forms. For other $^1O_2$ carriers, photo-oxidation should be preferred provided that the photosensitizer can be eliminated at the end of the reaction.

The interaction of $^1O_2$ with a naphthalenic compound (N) can be described by reactions (3.3) and (3.4). $^1O_2$ produced by a chemical or a photochemical source is either physically deactivated by N according to the rate constant k_q (see eqn (3.3)) or reacts with N according to the rate constant k_r (see eqn (3.4)). The overall reactivity of N towards $^1O_2$ can thus be expressed by the overall rate constant $k_t = k_r + k_q$ (see Table 3.1).

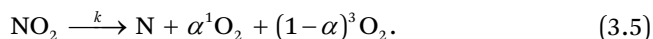


To explain the difference in reactivity of 1,4-substituted carriers (see Table 3.1) towards $^1O_2$, two factors have to be considered: the electron density of the naphthalene core and the steric hindrance induced by the 1,4 substituents themselves or by additional groups located at 2,3,5,8 positions. Electronic effects are of primary importance when a short spacer separates the hydrophilic groups from the naphthalene core. Thus, the electron-withdrawing effect of the quaternary ammonium group of **MNEA** or the OH functions of **NDMOL** leads to molecules 5 and 17 times less reactive than **MNP** respectively. Longer alkyl spacers increase the electron density of the naphthalene core but the steric hindrance lowers the rate of reaction with $^1O_2$ significantly. Thus, the overall rate constants ($k_r + k_q$) for **NDP** ($2.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) and for the more crowded **DHPN** ($1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) are significantly lower than the value for **MNP** ($7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$).

3.3.4. Thermal Release of $^1O_2$

The thermolysis of the endoperoxide NO_2 (see eqn (3.5)) follows a first-order kinetics with a rate constant k . The half-time of decomposition ($t_{50\%} = \ln 2/k$) or the time to decompose 95% of the starting endoperoxide ($t_{90\%} = \ln 20/k$) can be calculated. Table 3.1 indicates that most of the endoperoxides release 95% of their oxygen within 2 h at 37 °C. This value is convenient for carrying out biological tests. A part of the oxygen formed during the thermolysis is in the singlet excited state (see eqn (3.5)). This can be quantified by trapping with tetrapotassium rubrene-2,3,8,9-tetracarboxylate.^{28,40} Roughly speaking,

it can be considered that all naphthalenic carriers release $^1\text{O}_2$ in water with a 50% yield (see Table 3.1).



3.3.5. Substituent Effect on the Rate of Binding and Releasing $^1\text{O}_2$

3.3.5.1. Activating Effect of Oxygen Atom. The mesomeric activating effect of oxygen³⁶ (see Figure 3.7) was evaluated by grafting ether lateral side chains also bearing two hydroxyl groups. The activating effect of oxygen on the **DHPON** is remarkable ($k_q + k_r = 197 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$). Unfortunately, the formed endoperoxide is rapidly converted into an ester-aldehyde without releasing $^1\text{O}_2$, as already observed⁴¹ in the anthracene series.

When the oxygen is separated from the aromatic ring by one CH_2 segment ($R_1 = R_4 = \text{H}$, $R_2 = R_3 = \text{H}$, **14**), its inductive withdrawing effect results in a very low rate constant ($k_q + k_r = 0.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$). This constant is actually (4 times) smaller than that of the diol without side chains, *i.e.* **NDMOL** ($k_q + k_r = 0.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$). The poorly reactive compound **14**, although weakly hindered, leads to the 1,4- and 5,8-endoperoxides. Unexpectedly, the water-soluble tetra-amide **12** for which the aromatic ring is heavily hindered due to the presence of bulky side chains, reacts with $^1\text{O}_2$ to form the 5,8-endoperoxide besides the 1,4 ones in a ratio (30/70) in deuterated water at 25 °C (see Figure 3.8 and Table 3.4).⁴²

The photo-oxidation of **12** conducted at low temperatures (5 °C) to minimize thermolysis, revealed that the 1,4-endoperoxide ($k_r = 0.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) is formed 3 times faster than the 5,8-endoperoxide ($k_r = 0.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$). On the other hand, the half-time of decomposition of the two endoperoxides at 37° C are significantly different, *i.e.* $t_{1/2} = 7$ and 70 min, respectively.

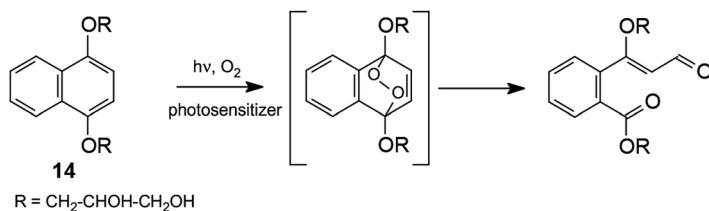


Figure 3.7. Mesomeric activating effect of oxygen during photo-oxidation leading to ester aldehyde.

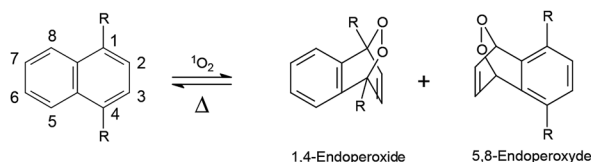


Figure 3.8. Reversible formation of 1,4 and 5,8-endoperoxides during oxidation of **10**.

Table 3.4. Half-time of decomposition ($t_{1/2}$) for 1,4- and 5,8-endoperoxides of **10** at 5, 25 and 37 °C, and overall ($k_r + k_q$) and chemical (k_r) quenching rate constants.

	$t_{1/2}$ (min)			$k_r + k_q$ ($10^6 \text{ M}^{-1} \text{ s}^{-1}$)	k_r ($10^6 \text{ M}^{-1} \text{ s}^{-1}$)
	5 °C	25 °C	37 °C		
1,4-Endoperoxide of 12	540	30	7	0.4	0.3
5,8-Endoperoxide of 12	Stable	300	70	0.4	0.1
NDPO ₂	Stable	113	23	2.8	1.4

Obtaining 5,8-endoperoxide seems possible in two cases: either the substituted ring is heavily hindered or the rate constant is low ($k_q + k_r = 0.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) but not too low, otherwise the reaction with $^1\text{O}_2$ does not take place. Unfortunately, the main disadvantage of tetra-amide **12** is its low reactivity towards $^1\text{O}_2$ (35 times less reactive than **NDP**). Thus, the synthesis at a gram scale of the corresponding endoperoxide is not feasible. Other amido-alcohols do not exhibit higher reactivity, as indicated by the overall quenching rate constants of tetra-amides **11** and **13**. On the other hand, the overall relatively higher quenching rate constants of the bulky tetrasalt **10** confirm that large side chains, attached to the naphthalenic core, cause too much steric hindrance to obtain sufficient reactivity towards $^1\text{O}_2$.

3.3.5.2. Activating Effect of One Methyl Group. More reactive compounds towards $^1\text{O}_2$ (compared to **NDP**) have been synthesized introducing water-solubilizing chains on positions 2 and 4, keeping one methyl group on position 1 for the activating effect. The general synthetic principle (see Figure 3.6) was applied by replacing 1-chloromethyl-4-bromomethylnaphthalene by 1-methyl-2,4-dichloromethyl naphthalene obtained by double chloromethylation of 1-methylnaphthalene.⁴³ After malonic synthesis or amidification, compounds **15–19** can be obtained. In particular **15**, with two carboxylate functions, is 4 times more reactive than its corresponding non methylated **NDP**. Similarly, the nonionic compound **17** is 8 times more reactive than **DHPN**.

In conclusion, addition of a methyl group in position 1 increases the reactivity by a factor of 4 to 8 in comparison with the nonmethylated 1,4-disubstituted corresponding naphthalenic compounds. However, the half-lifetime at 37 °C of 1-methyl-2,4-disubstituted naphthalenic endoperoxides (**15–19**) is comprised between 5 and 10 h, which is too long for delivery of $^1\text{O}_2$ in biological media. Thus, such trisubstituted naphthalene as **15** ($k_r + k_q = 10 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ in CH_3CN) may be used as $^1\text{O}_2$ trap rather than as $^1\text{O}_2$ carriers.^{39,43}

The grafting of a methyl group (compound **8**) in the α position of the amide group of **DHPN** was achieved by replacing diethyl malonate by methyl diethyl malonate in the general synthetic scheme (see Figure 3.6). But since this methyl group is too far from the naphthalenic core, there is no activating effect. The overall quenching rate constant of **8** is the same as those for **DHPN**.

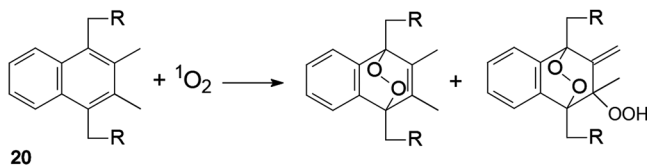


Figure 3.9. Oxidation of **20** leading to the formation of a mixture endoperoxide/hydroperoxide.

3.3.5.3. Activating Effect of Two Methyl Groups. Activation by 2 methyl groups at positions 2 and 3 was investigated by synthesizing compound **20**. This compound was obtained by double chloromethylation of 2,3-dimethylnaphthalene followed by malonic synthesis. The two methyl groups of **20** increase its reactivity towards $^1\text{O}_2$ by a factor 2 relative to the monomethyl derivative **15** and by a factor 8 relative to the nonmethylated derivative **NBP**. The half-lifetime at 37 °C of endoperoxide of **20** is $t_{1/2} = 63$ min. The photo-oxidation of **20** in the presence of $^1\text{O}_2$ leads initially to the usual 1,4 endoperoxide by [4+2] cycloaddition but unfortunately, the endoperoxide is oxidized further through the ene-reaction (see Figure 3.9). After total conversion of **20**, a 50/50 mixture of two oxidation products is obtained.

Purification at low temperatures allows the isolation of the 1,4-endoperoxide of **20** but during thermolysis at 37 °C, $^1\text{O}_2$ released reacts giving the hydroperoxide.

3.4. Biological Applications of Water-Soluble Carriers of $^1\text{O}_2$

Previously, it has been shown that a gentle warming of well-chosen naphthalenic endoperoxides at 37 °C for 2 h releases oxygen with 100% yield, half of which is in the excited singlet state, $^1\text{O}_2$, together with the parent hydrocarbon which is chemically inert. Therefore, they are mild nonphotochemical sources of $^1\text{O}_2$ that are suitable to generate well-defined amounts of pure $^1\text{O}_2$ in aqueous media.²⁹ They can be used for kinetic measurements or to distinguish among the oxidation products those deriving from $^1\text{O}_2$ or to accurately assess the cytotoxicity or biocidal activity of $^1\text{O}_2$. A special emphasis must be placed on the endoperoxides labeled with ^{18}O that allowed new light to be shed on the mechanistic features of the reaction of $^1\text{O}_2$ with various biological targets: fatty acids,⁴⁴ melatonin,⁴⁵ proteins,⁴⁴ nucleosides^{46–49} and DNA.^{44,50,51}

This section, divided into 5 subsections, focuses on the applications of $^1\text{O}_2$ carriers in biochemistry and biology. First, we discuss the kinetic issue that is of paramount importance for a short-lifetime species such as $^1\text{O}_2$. In the second subsection, we summarize within a table, all biologically relevant targets that have been confronted with one of the naphthalenic endoperoxides **NO₂**. The following three subsections detail three types of biologically relevant targets reactive towards $^1\text{O}_2$: molecules, macromolecules and micro-organisms.

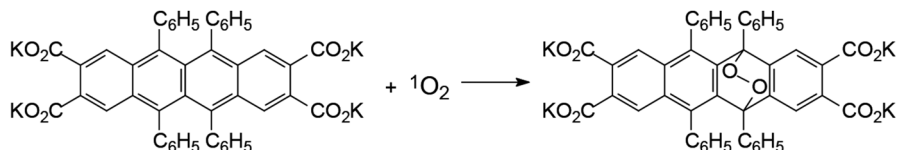
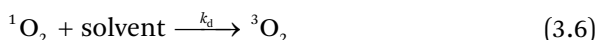


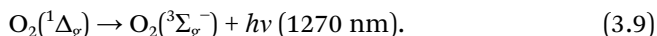
Figure 3.10. Sodium rubrene tetracarboxylate: a highly reactive and specific water-soluble trap of $^1\text{O}_2$.

3.4.1. Kinetics of $^1\text{O}_2$ Reactions with Biological Targets

Once generated in a medium, $^1\text{O}_2$ can interact with the solvent and the substrate (S) according to 3 competitive pathways (see eqn (3.6)–(3.8))⁵²



The chemical quenching (k_r) leads to the formation of product(s), whereas the physical quenching (k_q) only results in energy transfer and deactivation of $^1\text{O}_2$ into ${}^3\text{O}_2$. Physical quenching of $^1\text{O}_2$ by solvent can also take place with a rate constant k_d that is inversely proportional to the lifetime of $^1\text{O}_2$ (τ_Δ) in the solvent. The overall rate constants ($k_r + k_q$) can be determined accurately by laser flash photolysis but this technique does not allow differentiating k_r and k_q . The photochemical source of $^1\text{O}_2$ can be replaced by NO_2 and the progress of the reaction may be monitored either by detecting $^1\text{O}_2$ through its faint IR luminescence at 1270 nm (see eqn (3.9))⁵³ or by measuring, the cumulated concentration of $^1\text{O}_2$ with a water-soluble trap such as sodium rubrene tetracarboxylate which is both highly reactive and specific toward $^1\text{O}_2$ (see Figure 3.10).^{54,55}



As naphthalenic carriers release a precisely known amount of $^1\text{O}_2$, comparing the disappearance of the substrate in H_2O and in D_2O (in which τ_Δ is 16 times longer) allows access to the values of k_r and k_q separately.⁵⁶ When $^1\text{O}_2$ is produced in aqueous media, its reactivity towards a given biological target will depend on its rate constant with the reactive moiety of the target, the concentration of the target and the ability of the cell to deal with oxidative damage on this target. Only electron-rich substrates can compete with the solvent-deactivation pathway (see eqn (3.6)) since the lifetime of $^1\text{O}_2$ within the cell is probably less than 1 μs . This means that ($k_r + k_q$) must be much higher than $10^6 \text{ M}^{-1} \text{ s}^{-1}$. Figure 3.11 shows the rate constants ($k_r + k_q$), and k_r when it is known, of the main biological molecules found in the literature.

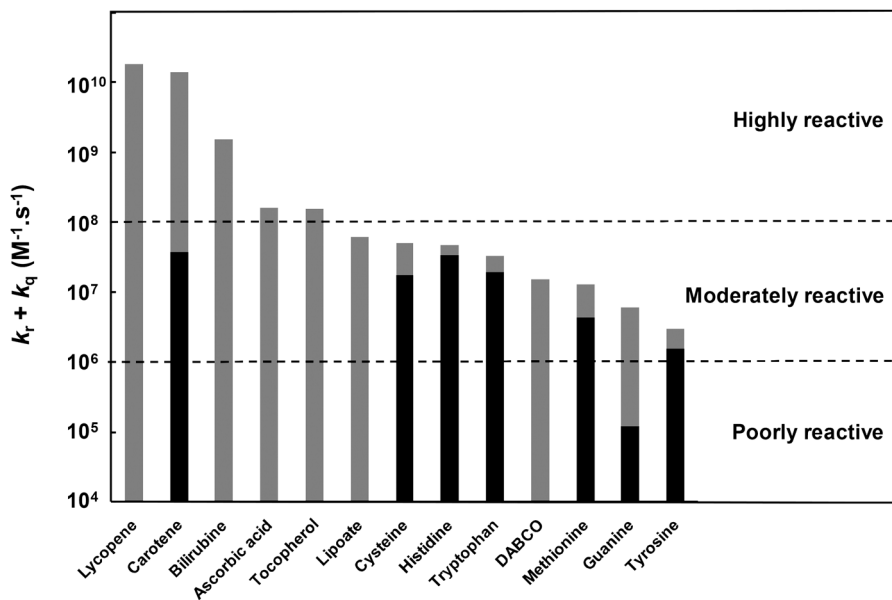


Figure 3.11. Reactivity of some biologically relevant molecules toward $^1O_2$. Gray bars: overall quenching rate constants ($k_r + k_q$); black bars: chemical quenching rate constants k_r when reported.

3.4.2. Biologically Relevant Targets of $^1O_2$ (Ref. 1)

$^1O_2$ is a strong electrophilic species exhibiting a peculiar bielectronic structure making it a powerful and selective oxidant.⁴ As a result, it chemically reacts with electron-rich organic compounds according to various well-established types of reactions: (i) the [4+2] cycloaddition with hydrocarbon or heterocyclic 1,3-dienes and polycyclic aromatics giving more or less stable endoperoxides (ii) the “ene” reaction with alkenes having an allylic hydrogen providing allylic hydroperoxides (iii) the [2+2] cycloaddition with alkenes devoid of allylic hydrogens or sterically hindered forming unstable 1,2-dioxetanes. It is worth noting that hydrocarbons with many conjugated double bonds do not chemically react with $^1O_2$ but preferentially interacts through physical quenching. Singlet oxygen transfers its energy to the substrate S giving ground-state oxygen $^3O_2$ and triplet excited state of the conjugated compound $^3S^*$. Singlet oxygen gives other specific reactions with phenols, sulfides, amines and heterocycles such as furans, pyrroles, indoles, imidazoles, purines, oxazoles, thiazoles and thiophenes. As a consequence, $^1O_2$ exhibits a high reactivity towards many biological targets including biologically relevant molecules (lipids, amino acids, phenolic antioxidants and various pharmaceutical or natural heterocycles), and biological macromolecules (proteins and nucleic acids). With a lifetime ranging from 100 ns to 1 μ s in biological systems, $^1O_2$ can travel some distance in the cell affording its interaction with biomolecules inducing cell damages.⁵⁷ Therefore, it has a strong cytotoxic activity and is a powerful biocide for viruses and bacteria. All the

Table 3.5. Biological targets tested with the water-soluble generators of $^1\text{O}_2$ **MNPO**₂, **NDPO**₂ and/or **DHPNO**₂.

Biologically relevant molecules
Lipids: squalene, ^{58,59} carotenoids, ^{60–65} fatty acids ^{44,59} Amines: spermine, ⁶⁶ spermidine, ⁶⁶ DABCO, ³³ aniline, ⁶⁷ <i>N,N</i> -dimethylanilines ^{15,68} Sulfides: thioanisol, ⁶⁹ methionine, ^{70,71} cysteine, ^{70,71} lipoate ⁷² Phenols and quinones: phenol, ⁶⁷ caffeic acids, ^{56,73} methyl gallate, ⁵⁶ trimethylhydroquinone, ⁵⁶ kinobeaon A, ⁶⁰ tocopherol (vitamin E), ^{60,61,74,75} trolox, ⁵⁶ flavonoids ⁷⁶ Heterocycles: tryptophan, ^{15,77} 5-hydroxytryptophan, ⁷⁸ bilirubin, ⁷⁹ MLCA (Cypridina luciferin analog), ^{80,81} coumarin, ^{82–84} dipyrone, ⁸⁵ aminopyrine, ⁸⁵ stobadine, ⁶⁶ melatonin, ⁴⁵ ascorbic acid (vitamin C), ⁸⁶ NAD, ⁷⁸ 8-oxodGuo, ^{84,6,47,87} deoxyguanosines ^{48,49,88,89}
Biological macromolecules
Nucleosides ^{8,46–49,78,87–89} oligonucleotides, ^{90–92} plasmid DNA, ^{66,70,72,90,93–99} DNA ^{17,29,100–108} Amino acids ^{70,71} and proteins, ¹⁰⁹ tyrosin phosphatase, ¹¹⁰ glucose 6-phosphate dehydrogenase ¹¹¹
Micro-organisms
Virus, ^{2,112} HIV, ^{8,35} Suid Herpes, ¹¹³ HSV1, ^{8,113,114} HCMV, ^{8,114} VSV, ¹¹⁴ adenovirus, ^{8,114} poliovirus ^{8,35,114} Bacteria: CC104, ¹¹⁵ <i>E. coli</i> ^{2,58,59,115–117} Cells: ¹⁰⁸ endothelial, ^{58,59} pancreatic, ¹¹⁸ prostate, ¹¹⁸ liver, ^{61,100,109} mitochondria of liver, ¹¹⁹ fibroblast, ^{8,120–122} T helper, ¹²³ mammalian, ⁹⁵ monkey, ¹⁰³ keratinocytes ⁸

biologically relevant targets that have been confronted to the water-soluble carriers of $^1\text{O}_2$ are listed in Table 3.5 and the chemical structures of the most relevant molecular targets are shown in Table 3.6.

3.4.3. Biologically Relevant Molecules Tested with Water-Soluble Carriers of $^1\text{O}_2$

“Dark” singlet oxygen generated from NO_2 chemically reacts with lipids such as squalene^{58,59} and unsaturated fatty acids^{44,59} to form lipid hydroperoxides according to the ene reaction. Thanks to their numerous conjugated double bonds, carotenoid pigments^{60–65} such as lycopene and β -carotene physically quench $^1\text{O}_2$ by energy transfer with high rate constants (10^{-9} – $10^{-10} \text{ M}^{-1} \text{ s}^{-1}$)¹²⁴ not far from a diffusion control process (see Figure 3.6). In the same way, tertiary amines such as DABCO³³ and electron-rich phenols such as tocopherol also physically quenches $^1\text{O}_2$ but through a different mechanism of electron transfer and with a lower rate constant (10^{-7} – $10^{-8} \text{ M}^{-1} \text{ s}^{-1}$). In particular, with NDPO_2 , it has been proved that the natural polyamines, spermine and spermidine that are ubiquitous in cells, may protect DNA against damage of $^1\text{O}_2$.⁶⁶ Thiols readily react chemically with $^1\text{O}_2$.¹²⁵ The kinetically favored reaction is with the deprotonated thiol, for cysteine thiolate, ($k_r + k_q$) = $1.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, while for the protonated thiol, ($k_r + k_q$) < $4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. Depending on the thiol concentration, products formed are either a disulfide or sulfonic acid. Methionine reacts with $^1\text{O}_2$ providing methionine sulfoxide that can further be oxidized to methionine sulfone. Tryptophan is a powerful $^1\text{O}_2$ scavenger, ($k_r + k_q$) $\approx 10^8 \text{ M}^{-1} \text{ s}^{-1}$ leading to the cleavage of the indole ring. The products of the reaction of tyrosine with $^1\text{O}_2$ are those expected from the reactions of the

Table 3.6. Biologically relevant molecules tested with the water-soluble generators of $^1\text{O}_2$ MNPO₂, NDPO₂ and/or DHPNO₂.

Lipids			
Squalene	Linoleic acid		
β-Carotene			
Amines			
Spermidine	Spermine	DABCO	Aniline
N,N-dimethylaniline			
Sulfides			
Thioanisole	Methionine	Cysteine	Lipoate
Phenols and Quinones			
Phenol	Caffeic acid	Methyl gallate	Trimethylhydroquinone
Trolox	Kinobean A	Tocopherol (Vitamin E)	
Heterocycles			
Tryptophan	5-Hydroxytryptophan	MLCA	Coumarine
Dipyrone	Aminopyrine	Stobadine	Melatonin
Bilirubine	Ascorbic acid (Vitamin C)	8-oxo-dG	Desoxyguanine

tyrosyl radical. Thus, it suggests that $^1\text{O}_2$ oxidizes tyrosine to form superoxide and the phenoxyl radical of tyrosine. The reactions of histidine with $^1\text{O}_2$ have been the subject of many studies. Histidine quenches $^1\text{O}_2$ both physically and chemically ($\approx 5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). However, since 75% is chemical quenching, it is thought that histidine is one of the major targets for $^1\text{O}_2$ attack on proteins. Most of the primary oxidation products formed by the reaction of biological molecules with $^1\text{O}_2$ have been shown to be unstable and evolve to give secondary oxidation products. The use of [^{18}O]-labeled $^1\text{O}_2$ released from thermolabile endoperoxides in association with HPLC-ESI-MS/MS analysis provides an elegant way to gain mechanistic insights into the formation and the decomposition pathways of initially generated peroxidic compounds.

3.4.4. *Biological Macromolecules Tested with Water-Soluble Carriers of $^1\text{O}_2$*

3.4.4.1. *Proteins (Ref. 109).* $^1\text{O}_2$ can interact with high-electron density proteins bearing double bonds or sulfur moieties by both physical and chemical quenching. The aromatic amino acids tyrosine and tryptophan as well as histidine and the sulfur-containing methionine and cysteine are primary sites of attack by $^1\text{O}_2$, while the aliphatic amino acids and peptide bonds do not react with a significant rate.^{70,71} Some authors have shown that the principal targets of $^1\text{O}_2$ in cells are the cellular proteins since damage to key proteins can leave the cell severely compromised.

3.4.4.2. *DNA (Ref. 7).* Because of its low redox potential, guanosine (2'-dG) is the DNA base that is most easily oxidized and consequently the most reactive with $^1\text{O}_2$. The reaction results in the oxygenation at carbon-8 giving 8-hydroxy-2'-deoxyguanosine (8-OHdG) that is 100-times more reactive with $^1\text{O}_2$. Strands breaks and alkali-labile sites appear on DNA molecules exposed to NDPO_2 .^{17,29,100-108}

3.4.5. *Micro-Organisms Tested with Water-Soluble Carriers of $^1\text{O}_2$ (Ref. 2)*

3.4.5.1. *Viruses (Ref. 2 and 112).* Pure $^1\text{O}_2$ generated from NDPO_2 effectively inactivates enveloped viruses (human immunodeficiency virus type 1,⁸ herpes simplex virus type 1,^{8,113,114} cytomegalovirus,^{8,114} vesicular stomatitis virus¹¹⁴) but has almost no effect on nonenveloped viruses (adenovirus^{8,114} and poliovirus 1^{8,35,114}). Nevertheless, this ionic carrier has no effect on *intra-cellular* viruses since it releases $^1\text{O}_2$ in the outer compartment of the cell. Considering the short lifetime of $^1\text{O}_2$ in biological media (100 ns–1 μs), $^1\text{O}_2$ generated out of the cell cannot reach intracellular targets. In contrast, the water-soluble and nonionic carrier, DHPNO_2 , can convey $^1\text{O}_2$ through lipid membranes and does not suffer electrostatic repulsion from negatively charged targets. Consequently, NDPO_2 inactivates only extracellular enveloped viruses, whereas DHPNO_2 exhibits virucidal activity on all types of viruses, enveloped (HIV) and nonenveloped (Poliovirus), extracellular and intracellular.³⁵

3.4.5.2. *Bacteria* (Ref. 2,58,59,115–117). Bacteria are significantly more resistant than enveloped viruses to $^1\text{O}_2$. However, the comparison of the biocidal activity of NDPO₂, DHPNO₂ and their parent hydrocarbons toward various bacteria shows that pure $^1\text{O}_2$ has an indisputable bactericidal activity. Gram-positive bacteria (*Enterococcus facium* and *Staphylococcus aureus*) are more responsive to $^1\text{O}_2$ than gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*).

3.4.5.3. *Cells* (Ref. 108). Chemically generated $^1\text{O}_2$ exerts genotoxic and cytotoxic effects. In addition, there is increasing evidence that singlet oxygen has pronounced effects on cellular signaling events leading to the induced expression of a variety of proteins.¹²⁶ The principal targets of $^1\text{O}_2$ in cells are the cellular proteins. From a kinetic point of view, DNA could appear as not an unimportant target (see Figure 3.6). However, it must be kept in mind that even though only a small fraction of the $^1\text{O}_2$ reacts with DNA, if this is not repaired and it is in a critical region, it could be devastating. The possibility of cell penetration afforded by DHPNO₂ has also been exploited for investigating the reaction of $^1\text{O}_2$ with intracellular DNA.¹²⁷

3.5. Conclusions

Water-soluble $^1\text{O}_2$ carriers are invaluable tools for determining nonambiguously and quantitatively the reactivity of $^1\text{O}_2$ toward complex biological targets. Contrary to the classical method based on sensitized photo-oxidation, they generate selectively under mild conditions (37 °C/2 h) known amounts of $^1\text{O}_2$ free from any other reactive species. However, two molecules of the endoperoxide precursor are required to generate a single molecule of $^1\text{O}_2$ unlike a photosensitizer that forms a large number of molecules of $^1\text{O}_2$ as long as it is irradiated in the presence of oxygen. Moreover, endoperoxides need to be prepared by reacting the naphthalene derivative with a primary source of $^1\text{O}_2$ ($h\nu/\text{sensitizer}/\text{O}_2$ or $\text{H}_2\text{O}_2/\text{MoO}_4^{2-}$). Accordingly, these carriers are suitable for conducting mechanistic studies and not to replace the current photochemical methods for organic synthesis or to treat cancerous tumors.

Three generations of carriers have been designed so far. The first carriers, MNP and NDP, bear anionic hydrophilic groups and release of known amounts of $^1\text{O}_2$ anywhere in aqueous solution. They are unable to cross lipid membranes and are electrostatically repelled by all compounds or micro-organisms negatively charged. They are well suited to estimate the proportion between the chemical (k_r) and the physical (k_q) quenching rate constants but they are less accurate than flash photolysis to determine the overall rate constant ($k_r + k_q$). Their main interest is mechanistic because they allow the oxidation products formed with $^1\text{O}_2$ to be distinguished from those derived from other ROS.

The second generation was developed to take into account the low $^1\text{O}_2$ lifetime in biological media (100 ns–1 s) and the short distance (<100 nm) it can travel

before being quenched into $^3\text{O}_2$. It is therefore essential that $^1\text{O}_2$ is formed near the target to avoid the loss of most $^1\text{O}_2$ molecules through physical quenching by H_2O or surrounding biological quenchers. The nonionic compound DHPN is the most representative of this family of carriers. It can approach charged targets and has been able to react with intracellular targets such as HIV virus.

The third generation consists of the same carrier but the endoperoxide [^{18}O]-DHPNO₂ is prepared with labeled oxygen ^{18}O . It allows for distinguishing between the multitude of oxidation products obtained from the reaction $^1\text{O}_2$ with complex biological targets those resulting from $^1\text{O}_2$ and those derived from $^3\text{O}_2$, or water or other ROS.

So far, none of the available carriers exhibit a strong affinity for a precise cellular target. The challenge still ahead is to graft a hydrophilic peptide or a nucleoside on the naphthalene core in order to selectively vectorize the carrier to the wanted target. In this case, $^1\text{O}_2$ would be released close to the target and would have a maximum impact with the minimum amount of endoperoxide while preserving other sites sensitive to $^1\text{O}_2$.

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Chapter 4

Production of Singlet Oxygen by Direct Photoactivation of Molecular Oxygen

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Molecular oxygen (O_2) has an uncommon electronic structure with two lower electronically excited states that are both spin singlet. Considering the free unperturbed molecule, transitions between one of these electronic states and the fundamental state are forbidden according to electric-dipole selection rules.^{1,2} While this so-called forbidden characteristic renders direct optical activation of singlet oxygen ($^1O_2$) very improbable, these weak transitions can be observed and furthermore they can be used to produce $^1O_2$ and to study its direct interaction with chemical acceptors and/or in biological systems.³ In this chapter, we will discuss $^1O_2$ direct production schemes that imply only a natural transition of molecular oxygen and that we name direct photoactivation (DPA). Our purpose will be to highlight that despite the low efficiency of DPA compared to nondirect production methods (DPA is *ca.* 10^4 – 10^5 times less efficient than photosensitized production), it can be seen as a powerful tool to study chemistry and biology of $^1O_2$. Indeed the simplicity of DPA makes the $^1O_2$ production rate only sensitive to O_2 concentration while other techniques (such as photosensitized production) can have complex dynamics. Moreover, indirect $^1O_2$ production pathways can be invasive for instance in biological systems.

The first section is dedicated to a brief history of the discovery of the O_2 electronic transitions under consideration in this chapter. We will briefly review pioneering works that have allowed a better understanding of peculiar spectroscopic properties of O_2 . This will allow us to introduce DPA schemes of $^1O_2$.

It has been reported that infrared laser radiation cause cytotoxic effects *in vitro* and *in vivo*.^{4–8} Only some of these effects could be unambiguously attributed to DPA of $^1O_2$.^{7,8} In this context, quantitative informations on DPA of $^1O_2$ in natural systems (at room temperature and atmospheric pressure) is important both for the study of $^1O_2$ in biochemical systems and for biomedical applications of infrared lasers. In the second section we will focus on recent work regarding DPA of $^1O_2$ in liquid solvents at standard temperature and pressure (STP). These studies have led to estimation of absorption cross sections of O_2 bands in homogeneous liquids as well as in heterogeneous solutions.

In the third section we will discuss the use of DPA as an alternative scheme to traditional photodynamic therapy (PDT) for destruction of biological cells or tumors. First, we will review reports of cytotoxic effects possibly attributed to DPA of $^1O_2$ in biological systems including *in vivo* tumors. Then, we will discuss recent studies showing that cell death can be induced *via* DPA of $^1O_2$ in cultured mammalian cells. Finally, we will shed light on the high potential of DPA of $^1O_2$ for therapeutic use and for more fundamental work to better understand oxidative stress in the cellular response.

4.1. Introduction: Absorption Bands of Molecular Oxygen and Singlet Oxygen Production

Molecular oxygen has quite unusual spectroscopic properties that arise from the fact that the ground state is a spin triplet and the two first excited electronic states are spin singlet. The first electronic state ($^1O_2$) with spectroscopic

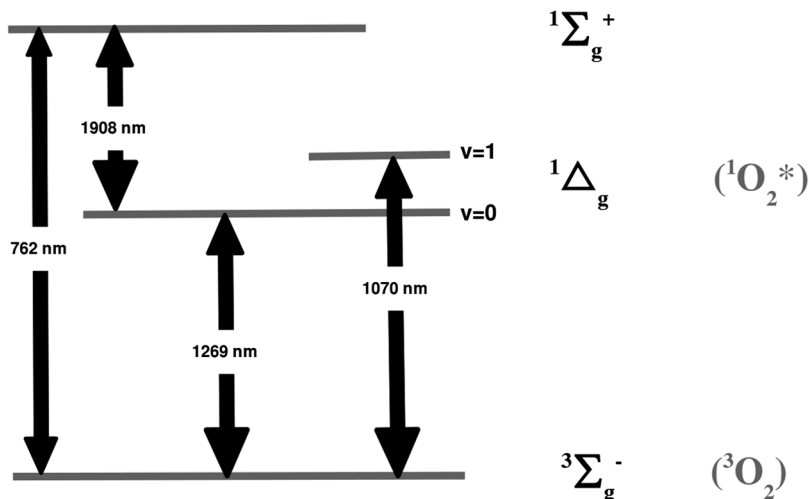


Figure 4.1. Spectroscopic properties and electronic states of molecular oxygen. $^3\Sigma_g^-$ is the ground state. $^1\Delta_g$ is the first excited electronic state. Only the two vibronic states ($v = 0$ and $v = 1$) are mentioned. $^1\Sigma_g^+$ is the second excited electronic state.

notation $^1\Delta_g$, lies *ca.* 94 kJ mol^{-1} above the ground state $O_2 (^3\Sigma_g^-)$, whereas the second singlet state ($^1\Sigma_g^+$) is located *ca.* 156 kJ mol^{-1} above the ground state. Figure 4.1 shows a schematic representation of molecular oxygen electronic states and associated transitions wavelengths.

For the unperturbed molecule, transitions between one of these electronic states and the fundamental state are forbidden according to electric-dipole selection rules and the two excited electronic states are metastable. Their respective pure radiative lifetimes are calculated to be 72 min for $^1\Delta_g$ and 11 s for $^1\Sigma_g^+$.⁹

4.1.1. Early Observation of Forbidden Transitions of Oxygen

Optical transition from ground-state molecular oxygen ($^3\Sigma_g^-$) to its second electronically excited state ($^1\Sigma_g^+$) is responsible for the dark band at *ca.* 765 nm in the solar spectrum observed by Wollaston in 1802 and Fraunhofer in 1817.¹ This feature known as the Fraunhofer A line was later named the atmospheric band of O_2 .¹ The interpretation of this band is one of the successes of molecular orbital theory. Indeed in 1928 Mulliken has not only discovered the $^1\Sigma_g^+$ state of O_2 but he also predicted the existence of another singlet state of lower energy: the first electronically excited state of molecular oxygen ($^1\Delta_g$), so-called singlet oxygen ($^1O_2$). Transition from O_2 to $^1O_2$ occurs at a wavelength of *ca.* 1270 nm and it has been observed in absorption of liquid oxygen a few years later by Ellis and Kneser.¹⁰ The fine structure of this band was then resolved and assigned in the solar spectrum by Herzberg.¹¹ Fifteen years later Herzberg and Herzberg reported the observation of a *ca.*

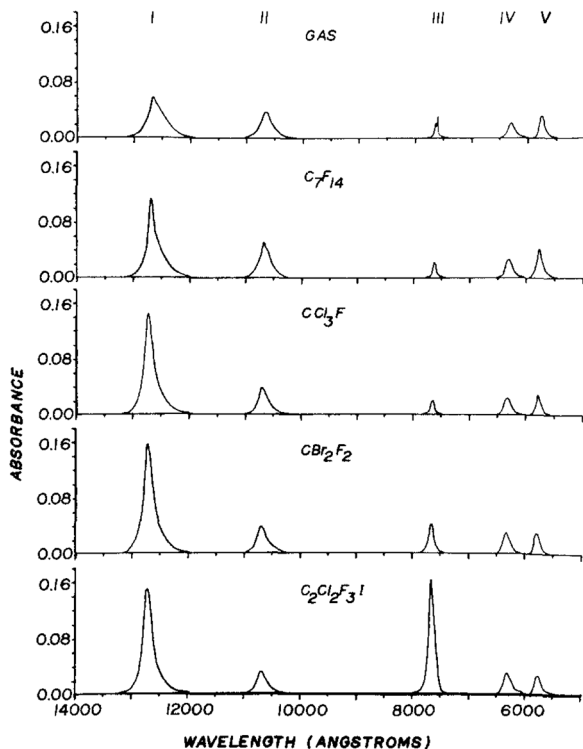


Figure 4.2. Absorption spectra of molecular oxygen in various solvents at high oxygen pressure. Reprinted with permission from C. Long and D. R. Kearns, *J. Chem. Phys.*, 1973, **59**, 5729. Copyright 1973, AIP publishing LLC.

1070 nm absorption band corresponding to the transition from O_2 to $^1O_2$ in its vibrational ($\nu = 1$) excited state.¹²

4.1.2. Solvent Effect on Molecular Oxygen Absorption Bands

These transitions were observed in absorption at high oxygen concentration and in the presence of other molecular species (solvents) in gas,^{13,14} liquids^{2,13} and solids.¹⁵ In Figure 4.2, one will easily identify the 1270 nm band associated to a $^3\Sigma_g^- \rightarrow ^1\Delta_g$ ($\nu = 0$) transition and the 765 nm band associated to the $^3\Sigma_g^- \rightarrow ^1\Sigma_g^+$ transition and the 1070 nm band associated to the $^3\Sigma_g^- \rightarrow ^1\Delta_g$ ($\nu = 0$). One can also notice the presence of two other bands at *ca.* 580 nm and *ca.* 630 nm, both of them being associated with simultaneous excitation of two oxygen molecules.^{1,2}

In pure oxygen at low density and in the gas phase, the 765 nm band intensity varies linearly with oxygen pressure and it can be reasonably described as a magnetic-dipole transition.² However, the 1270 nm, 1070 nm, 580 nm and 630 nm band intensities exhibit square dependence with oxygen pressure.

While this is expected for the bimolecular processes associated with the 580 nm and 630 nm bands, this feature cannot be understood for the 1270 nm and 1070 nm bands by only considering the unperturbed molecule. Indeed, this square dependence of the 1270 nm and 1070 nm absorptions arises from molecular interactions that mix states, thus introducing allowed characters into forbidden transitions for the unperturbed molecule.² Moreover, this intermolecular enhancement is also observed *via* interactions with another molecular species (solvent) and transition rates can rise to *ca.* 104 times in absorption as well as in emission (symmetric process).^{2,9} The solvent effect on radiative process of molecular oxygen gave rise to a number of experimental and theoretical studies.^{2,16,17}

4.1.3. Production Schemes for Direct Photoactivation of Singlet Oxygen

One can now envision several DPA schemes of $^1\text{O}_2$ that are summarized on Figure 4.3. Note that processes involving two oxygen molecules will not be considered any further as we will mainly focus $^1\text{O}_2$ DPA in solvents.

The first production scheme is based on the 1270 nm photon absorption by O_2 leading to direct activation of $^1\text{O}_2$. As one can see in Figures 4.2 and 4.3, this is the simplest and most efficient DPA of the $^1\text{O}_2$ scheme in most solvents. Radiation around 1070 nm is also expected to lead to $^1\text{O}_2$ formation as the vibrational excited state ($\nu = 1$) will be rapidly converted to the $\nu = 0$ state by heat release. The last scheme at 765 nm involves a transition toward the $^1\Sigma_g^+$, which is known to rapidly decay into the $^1\Delta_g$ ($\nu = 0$) state because of collisions between oxygen and solvent molecules.⁹ Krasnovsky and coworkers recently reported relative $^1\text{O}_2$ production rate of these three

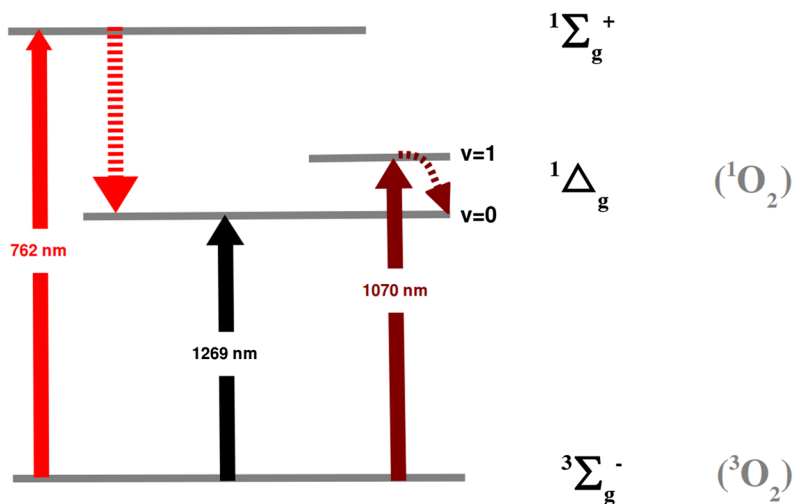


Figure 4.3. Different singlet oxygen direct photoactivation pathways. Three pathways are considered at 1270 nm (black), 1070 nm (dark red) and 765 nm (red).

schemes.¹⁸ The rate of $^1\text{O}_2$ production following 765 nm and 1070 nm radiation are reported to be, respectively, 3.5 and 70 times less efficient than under 1270 nm irradiation.

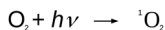
4.1.4. Detection of Singlet Oxygen

In order to use $^1\text{O}_2$ DPA to study either properties of $^1\text{O}_2$ in liquids or its interactions with other species in biological systems, one needs to estimate the $^1\text{O}_2$ production rate and/or reactivity. We will discuss here two methods that have been used to detect $^1\text{O}_2$ and to quantify its production rate following DPA. We will start by describing $^1\text{O}_2$ detection *via* its phosphorescence at 1270 nm. Estimates of the $^1\text{O}_2$ production rate following DPA in liquid solvents at STP were obtained using a chemical acceptor. We will describe here the principles of these methods.

4.1.4.1. Photodetection of Singlet Oxygen. Krasnovsky was the first to report direct detection of phosphorescence at 1270 nm of dye-photosensitized $^1\text{O}_2$ in CCl_4 solutions.^{19,20} Later, Khan and Kasha reported such a direct observation in liquid solutions including water at room temperature.²¹ In this type of experiment, $^1\text{O}_2$ is produced by photoactivation of sensitizing dye at a wavelength (usually in the UV or visible range) that is well separated from the $^1\text{O}_2$ infrared emission at 1270 nm. Excitation light and phosphorescence signals can be separated by appropriate dichroic and bandpass spectral filters.

This type of technique was also used to detect $^1\text{O}_2$ phosphorescence following DPA at 1070 nm. First, Skuja and Güttler reported detection of interstitial oxygen molecules in SiO_2 glass.²² Using a Nd-YAG 1064 nm laser associated with a Fourier transform infrared spectrometer, they were able to separate $^1\text{O}_2$ 1270 nm luminescence from resonance Raman scattering signal. Then, Jockusch and coworkers reported time-resolved detection of $^1\text{O}_2$ phosphorescence following powerful pulsed YAG-laser activation in condensed oxygen at 77 K.²³ They were able to detect not only 1270 nm phosphorescence but also a transition involving bimolecular processes in the visible region.

Simultaneous DPA and direct spectroscopic observation of $^1\text{O}_2$ in liquid solvents at STP have never been reported. Anquez and coworkers tried to create $^1\text{O}_2$ *via* a 765 nm nanosecond laser pulse and to detect subsequent 1270 nm emission in liquid solvents at room temperature (data not published). They estimated that using a pulse energy at 765 nm of *ca.* 10 mJ, the amount of $^1\text{O}_2$ created is one order of magnitude below the detection limit in their experimental conditions. In their experiment, the detection limit was dictated by the poor efficiency of detectors in the infrared region (Hamamatsu PMT, quantum efficiency *ca.* 2%). However, using newer sensors with higher quantum yield (25%), the detection limit would be improved by the missing 10-fold. Moreover, using the 1270 nm DPA scheme together with a gated



Scheme 4.1. First reaction involved in chemical detection of singlet oxygen. Irradiation at appropriate wavelength (1270 nm; 1070 nm or 765 nm) leads to ${}^1\text{O}_2$ formation.



Scheme 4.2. Second reaction involved in chemical detection of singlet oxygen. ${}^1\text{O}_2$ reacts with the chemical acceptor T, and lead to the formation of endoperoxide TO_2 .

detector (the detection gain of which can be shut down during laser pulse excitation), one can expect to further improve the detection limit and render possible simultaneous DPA and direct spectroscopic observation of ${}^1\text{O}_2$ in liquid solvents.

4.1.4.2. Chemical Detection of Singlet Oxygen. The pioneer works of Evan and Matheson showed that red and infrared light causes formation of ${}^1\text{O}_2$ and oxygenation of ${}^1\text{O}_2$ chemical traps in solvents saturated with oxygen at high pressure (130–150 atm).^{24,25} Later, Krasnovsky and coworkers provided experimental proof of DPA of ${}^1\text{O}_2$ in liquid solvents at STP. ${}^1\text{O}_2$ was detected *via* the oxidation 1,3-diphenylisobenzofuran (DPIBF) and tetracen.^{3,26,27}

As will be discussed in Section 4.2, such chemical acceptors have been used to estimate ${}^1\text{O}_2$ production rate under DPA in liquids at STP. Let us for now expose the principle of ${}^1\text{O}_2$ detection following DPA with a chemical acceptor.

The photo-oxygenation process of a chemical trap (T) such as DPIBF or tetracen, occurs through the following reactions (Schemes 4.1 and 4.2): where $h\nu$ represents the energy of a photon at a wavelength corresponding to ${}^1\text{O}_2$ DPA schemes described in Figure 4.3 (1270, 1070 or 765 nm) and TO_2 is the oxygenated trap. The trap T exhibits an absorption band in the visible region (*ca.* 405 nm for DPIBF) while the oxygenated trap TO_2 is colorless. Thus, measuring the visible absorbance of an aerobic solution containing trap prior and after irradiation at 1270 nm, 1070 nm or 765 nm gives an estimation of the ${}^1\text{O}_2$ production rate.²⁸

4.2. Direct Photoactivation in Liquids at Standard Temperature and Pressure

In solvent saturated with air at normal atmospheric pressure, O_2 absorption bands are hidden by solvent proper absorption and light scattering. Therefore, these lines cannot be detected by conventional infrared spectrometers. Instead, scientists used the chemical acceptor method in order to detect ${}^1\text{O}_2$ DPA in liquids and to measure the ${}^1\text{O}_2$ production rate. This has been achieved in the pioneering work of Krasnovsky's group who provided evidence for

DPA and detection $^1\text{O}_2$ in liquid solvents (including water) at STP.^{3,26,27} In this section we will propose a comprehensive overview of the detection of $^1\text{O}_2$ produced by DPA in solvents at STP and we will present recent progress in the measurement of DPA $^1\text{O}_2$ production rate.

All the studies mentioned in this section have benefited from some technological improvements in continuous-wave infrared lasers emitting around 1270 nm, we will start with a brief overview of the development of these new sources. Then we will discuss proof of $^1\text{O}_2$ DPA in liquid solvents and we will expose methods to measure the $^1\text{O}_2$ production rate and reactivity. Finally, we will discuss recent works on DPA of $^1\text{O}_2$ in heterogeneous solutions that we envision as a basis for the study of $^1\text{O}_2$ interactions in more complex environments such as biological media.

4.2.1. New Laser Sources for Direct Photoactivation of Singlet Oxygen

The investigation of the direct creation of singlet oxygen through the transition $^3\Sigma_g^- \rightarrow ^1\Delta_g$ ($v = 0$) of oxygen molecules in solvent at STP requires very specific laser sources. As the transition is forbidden at the electric-dipole approximation, it is obviously preferable that those laser sources deliver a high optical power, typically greater than hundreds of milliwatts. The specific wavelength of 1270 nm characterizing this transition falls in a particular wavelength region located between the end of the visible range (*ca.* 800 nm) and the fiber telecommunication window (*ca.* 1450 nm). From an historical point of view, this wavelength range has not been greatly exploited for practical applications and it is not easy to find commercial high-power laser sources having wavelengths that fall around 1270 nm.

In this context, various particular laser sources have been developed by people having an interest in studying the direct creation of singlet oxygen for medical applications or for more fundamental studies of the 1270 nm transition of oxygen molecules in solvents. For instance, a laser manufactured at the Russian Cancer Center in 2003 consisted of an array of continuous GaAlAs diode lasers.³ The light emitted by the laser diodes is focused inside an optical fiber. With this system, an optical power of *ca.* 55 mW has been obtained at a wavelength of *ca.* 1266 nm.³ Quantum well GaAlAs lasers operating at a wavelength of 1269 nm have been also manufactured by the “Medical innovative technologies” firm (Moscow).²⁹ Another laser system, assembled at the Institute of Physics of the Russian Academy of Sciences in 2003, consisted of a wavelength tunable forsterite laser pumped by a Nd:YAG laser with an acousto-optical Q-switch operated at a 25 kHz repetition rate.³ With this laser system, an infrared radiation with 5 nm bandwidth and 30–150 mW power has been obtained at 1200–1290 nm. Note that both continuous and mode-locked chromium-doped forsterite lasers have also been developed at wavelengths around 1270 nm.^{30,31}

Compared with traditional solid-state lasers, fiber lasers emerged around the beginning of the 2000s as light sources presenting many significant

advantages in terms of compactness, reliability and flexibility. In particular, continuous-wave pumped all-fiber Raman lasers are versatile light sources that are virtually able to deliver high-power radiation at any wavelength across the 1–2.1 μm spectral region.^{32–34} Raman fiber lasers (RFLs) have also been shown to exhibit many attractive capabilities such as multiwavelength operation, pulsed emission or tunability.^{35–37} Nowadays, RFLs have many applications in various fields such as fiber sensing, fiber telecommunications, material processing but also in surgery and in the treatment of some oncological diseases.^{6,38}

Taking advantage of the potentiality and of the versatility of RFLs, Anquez and coworkers have designed a tunable RFL especially for the investigation of the 1270 nm transition of molecular oxygen. This laser was designed and demonstrated in 2010.⁷ A phosphosilicate fiber having a large Raman shift of *ca.* 40 THz has been used instead of conventional germanosilicate fibers having a Raman shift of *ca.* 13.3 THz. With this fiber, laser emission is obtained around 1270 nm from only one Stokes cascade that is initiated by a commercial pump laser operating around 1060 nm. The RFL oscillates in a ring cavity made from commercially available fiber components. Wavelength tunability of the RFL is obtained by using a tunable pump laser and by taking advantage that the ring cavity does not incorporate wavelength-selective components. Tunability of this RFL has been demonstrated between 1240 nm and 1289 nm. The laser delivers a total power of 2.5 W at the maximum available pump power of 7 W.⁷

4.2.2. *Proofs of Direct Optical Creation of Singlet Oxygen in Liquid Solvents*

In order to obtain evidence of $^1\text{O}_2$ after DPA at 1270 nm and 1070 nm, Krasnovsky and coworkers measured the absorbance of aerobic solutions at STP (in which a $^1\text{O}_2$ trap was dissolved) before and after irradiation of the solution at the appropriate wavelength (see Section 4.1.4.2). They used DPIBF and tetracen as chemical $^1\text{O}_2$ acceptors.^{3,26,27} They observed a *ca.* 10% decrease of the 411 nm absorbance of ethanol solutions with *ca.* 40 μM DPIBF after 10 min irradiation at 1266 nm with a laser power of 55 mW.³ This corresponds to a *ca.* 4 μM decrease of DPIBF concentration. The effect of laser radiation increased linearly with oxygen concentration in the solution and it was strongly inhibited by addition of known $^1\text{O}_2$ quenchers.³ Furthermore, the infrared laser action spectrum of trap photo-oxygenation clearly coincides with the spectrum of $^1\text{O}_2$ phosphorescence in various solvents.²⁶ One should notice that the control experiments mentioned above should be carefully done if another O_2 band and/or another chemical species are to be investigated.

The 1270 nm absorption cross section of O_2 dissolved in CCl_4 at STP was estimated to be $\sigma_{1270} \sim 10^{-23} \text{ cm}^2$.²⁸ This value is in good agreement with the value that can be extrapolated from experiments performed at very high oxygen pressure (>20 atm).^{14,26} Final evidence supporting trap

photo-oxygenation *via* DPA of $^1\text{O}_2$ was obtained by showing that 1070 nm radiation induced a photo-oxygenation rate *ca.* 100 times slower compared to 1270 nm light, which correlates with the Franck–Condon factor for this vibronic transition.³

4.2.3. Production-Rate Measurement of Singlet Oxygen

After this success, the Russian group investigated spectroscopic properties of the $^3\Sigma_g^- \rightarrow ^1\Delta_g$ ($\nu = 0$) band at 1270 nm and the $^3\Sigma_g^- \rightarrow ^1\Sigma_g^+$ at 765 nm in various solvents.^{18,28,29} In particular, they were able to obtain O_2 absorption spectrum and they were also able to estimate O_2 absorption cross section for these transitions. These data were later corroborated.^{7,39}

In order to reliably estimate $^1\text{O}_2$ production rate with the chemical acceptor method, one obviously needs to know the reaction rate, k_T , of $^1\text{O}_2$ with the trap as well as the $^1\text{O}_2$ lifetime, τ_Δ^0 in the solvent in the absence of this trap. In other words, one needs to estimate what is the fraction of $^1\text{O}_2$ that reacts with the trap. The k_T data for most $^1\text{O}_2$ chemical acceptor and the estimates of τ_Δ^0 in several solvents are quite scattered in the literature that could lead to difficult interpretation of the data.

On the one hand, the Russian group further developed the technique using careful comparison of the trap photo-oxygenation rate induced by DPA of $^1\text{O}_2$ with the trap photo-oxygenation rate under photosensitized production of $^1\text{O}_2$.¹⁸ Measuring the quantum yield of singlet oxygen generation by the photosensitizer in a separate experiment, it is possible to obtain reliable results.¹⁸ In all the studied solvents, the absorption cross section for the 1270 nm band strongly depends on solvent and it correlates with the pure $^1\text{O}_2$ radiative lifetime as reported previously.^{14,18} The 765 nm band exhibits weaker solvent dependence.¹⁸

On the other hand, motivated by the same idea of improving the chemical acceptor detection method, Courtade and coworkers have developed a kinetic analysis of photo-oxygenation of traps by $^1\text{O}_2$ DPA.³⁹ They have studied the 1270 nm O_2 band in various solvents. In their experiments, the evolution of the 405 nm absorbance is monitored in real time in the solvent. They have shown that the trap disappearance rate V , is described by the following equation:

$$V = \frac{\Gamma}{\frac{\beta}{[T]} + 1} \quad (4.1)$$

where $\beta = (\tau_\Delta^0 \times k_T)^{-1}$ is named the reaction index and Γ is the $^1\text{O}_2$ production rate upon DPA.

Figure 4.4 shows the experimental data in acetone and acetone d_6 . In most solvents one can observe two regimes for the reaction rate. When $[T]$ is large compared to β the reaction rate saturates and equals Γ while if $[T]$ becomes

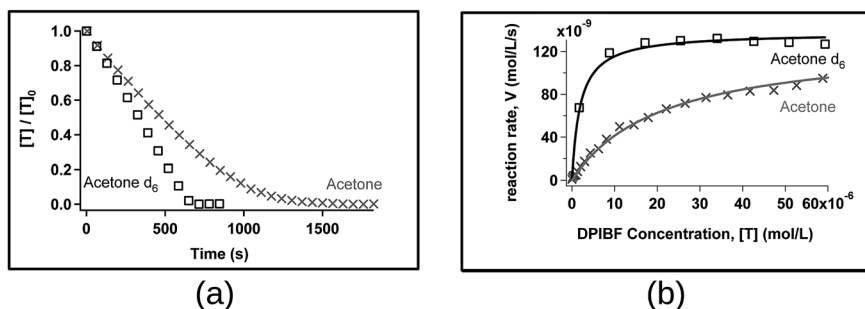


Figure 4.4. Reaction kinetics of $^1O_2$ and chemical trap T. (a) Temporal evolution of DPIBF concentration upon *ca.* 1.1 W irradiation at 1270 nm in air-saturated solutions of acetone (gray cross) and acetone d_6 (black squares). Initial concentration of DPIBF is $80 \mu\text{mol L}^{-1}$ for both solvents. (b) Trap disappearance rate calculated from experiments performed in (a). The symbols for acetone and acetone d_6 are kept the same. Data are fitted with eqn (4.1) and the fitted results are represented with continuous lines. One can find $\Gamma = 1.29 \times 10^{-7} \text{ mol L}^{-1} \text{ s}$ and $\beta = 1.8 \times 10^{-5} \text{ mol L}^{-1}$ for acetone and $\Gamma = 1.38 \times 10^{-7} \text{ mol L}^{-1} \text{ s}$ and $\beta = 1.78 \times 10^{-6} \text{ mol L}^{-1}$ for acetone d_6 .

smaller the reaction rate varies linearly as $[T]$ increases with a slope Γ/β . Therefore, experimental data can be fitted with eqn (4.1). Γ and β can thus be estimated simultaneously and unambiguously without any prior knowledge on the system. Note that this is only true when the two regimes can be observed, *i.e.* T can be dissolved at high enough concentration in the solvent. Both methods reported in ref. 18 and 39 give consistent results regarding the 1270 nm O_2 band in various solvents.

4.2.4. Direct Photoactivation of Singlet Oxygen in Heterogeneous Solutions

Sivéry and coworkers performed experiments in which the nonwater-soluble DPIBF is encapsulated in lipid nanocapsules (LNCs) dissolved in D_2O . They use this system to monitor the formation of singlet oxygen in these microheterogeneous systems.⁴⁰ A two pseudophase kinetic model for $^1O_2$ distribution is applied to LNCs dispersions in D_2O . As shown in Figure 4.5 the kinetics of photo-oxygenation is well described with a simple analytical expression similar to eqn (4.1). In this context one can extract the apparent production rate and apparent reaction index that vary linearly with LNC volume fraction.⁴⁰ From these data 1270 nm absorption cross section of O_2 could be extrapolated to pure deuterated water.

This study could be of interest in living cells in order to estimate singlet-oxygen production for dosimetry and reactivity with specific biotargets. Moreover, LNCs dispersions in D_2O are a heterogeneous medium that can mimic a lipid membrane environment. The two pseudophases model could then represent a first step for a better understanding of the dynamic of singlet oxygen in living cell.

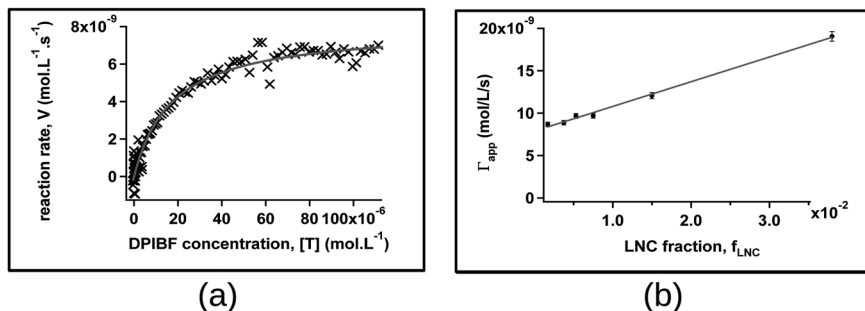


Figure 4.5. Reaction kinetics of $^1\text{O}_2$ and chemical trap T in heterogeneous (LNC/ D_2O) solutions. DPIBF is encapsulated in LNCs suspended in D_2O . (a) DPIBF disappearance rate as a function of DPIBF concentration upon 1270 nm irradiation at *ca.* 1.1 W for a LNC volume fraction of 0.0017 in D_2O . The apparent reaction rate is well described by eqn (4.1) (continuous line). One can find the apparent $^1\text{O}_2$ production rate of $\Gamma = 7.95 \times 10^{-9} \text{ mol L}^{-1} \text{ s}$ and an apparent reactivity index $\beta = 17.3 \times 10^{-6} \text{ mol L}^{-1}$. (b) Apparent $^1\text{O}_2$ production rate exhibits a linear dependence with LNC volume fraction. From these data one can extrapolate $^1\text{O}_2$ in pure D_2O .

4.3. Direct Photoactivation in Biological Systems: A New Tool for Photobiology?

While DPA of $^1\text{O}_2$ has been proposed to be involved in the stimulating and healing effect associated with low intensity lasers, other hypotheses have also to be considered.^{41,42} We will give with a brief review of *in vitro* and *in vivo* cytotoxic effects reported upon irradiation wavelengths matching $^1\text{O}_2$ DPA schemes. Then, we will present recent work showing that, under certain conditions, cell death can be induced by the sole action of $^1\text{O}_2$ DPA. Finally we discuss how, in our opinion, DPA of $^1\text{O}_2$ opens up new opportunities for the study of oxidative stress and for therapeutic use.

4.3.1. Overview of the Light Oxygen Effect on Biological Systems

As a result of the poor efficiency of the PS-free (photosensitizer-free) production of $^1\text{O}_2$ (see Section 4.2), only a few investigations about cytotoxic effects arising from 1270 nm irradiation have been undertaken in biological systems. For instance, Zakharov and Ivanov have discussed the plausible role of singlet oxygen in reversible changes of cellular membranes and in growth inhibition of rat tumors upon 1264 nm irradiation.⁴ The recent development of high-power reliable laser sources around 1270 nm have allowed a new interest in a PS-free 1270 nm laser irradiation of living cells in order to induce oxidative stress. A high-power Raman fiber laser has been used to treat human basalioomas leading to a destruction of the tumor tissues.⁶

Whereas the irradiation wavelengths in all these works coincide with O_2 bands, the direct and sole action of $^1\text{O}_2$ could not be demonstrated

unambiguously. In this context we designed experiments in order to determine in which conditions DPA $^1\text{O}_2$ can induce cytotoxic effect leading to cell death without the implication of other cytotoxic effects.⁴³ These data are discussed in Section 4.3.2. Later using pulsed quantum-dot laser diodes emitting at 1268 nm, singlet oxygen has been created in living cells.⁸ The authors take here advantage of the simplicity of the DPA production scheme to measure both production of superoxide anion and cytosolic calcium in various cell types. With these data the authors build up a kinetic model to describe rapid reactive oxygen species scavenging by the cellular antioxidant system.

4.3.2. Cell Death Induced by Direct Photoactivation of Singlet Oxygen

Obviously high laser powers have to be used in order to induce cell death by DPA of $^1\text{O}_2$ only. High-power lasers might induce temperature increase that can produce cytotoxic effects leading to cell death. With this in mind, Anquez and coworkers performed around 1270 nm irradiation of cultured MCF7 under conditions for which the laser-induced temperature do not exceed 37 °C.^{43,44} In these conditions, as shown on Figure 4.6, the cell death action spectrum of laser irradiation matches the absorption spectrum of O_2 . The amount of cell death correlates with oxygen concentration in the culture medium and in particular cell death could be totally inhibited if oxygen

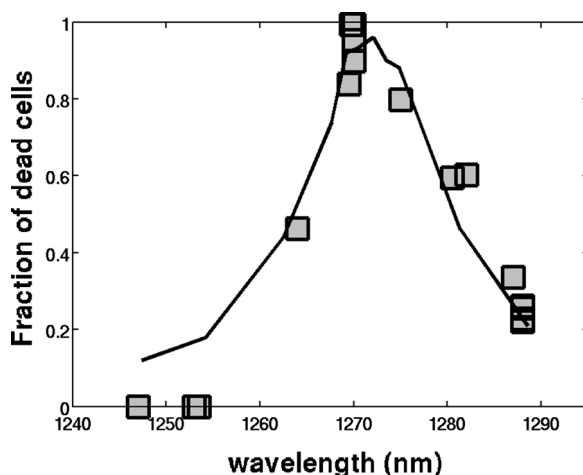


Figure 4.6. Cell death action spectrum of 1270 nm irradiation. MCF7 cells are irradiated with a tunable laser from 1247 nm to 1289 nm. Irradiation lasts for 3 h with a 100 mW laser power with beam diameter of 300 μm FWHM. These experiments are performed in a custom-made incubator allowing time-lapse microscopy. Cell death is assessed 24 h after irradiation. The incubator is maintained at 25 °C during irradiation and restored to 37 °C after laser treatment. In these conditions, the temperature does not exceed 37 °C even in the presence of the laser. The fraction of dead cells (squares) is given for a circular region having a radius of 200 μm centered around the laser spot. This death action spectrum correlates well with O_2 absorption spectrum measured in ethanol (continuous line).

was temporary removed from the medium. Moreover, extracellular as well as intracellular quenchers of $^1\text{O}_2$ significantly inhibit cell death.^{43,44}

Importantly, Anquez and coworkers found that, in their experimental conditions, cellular fate depends on a characteristic fluence threshold: if cells are exposed to a sufficient amount of 1270 nm light they died, while the others survived. They measured a fluence threshold of $100 \text{ W cm}^{-2} \text{ h}$ and estimation of the minimal amount of $^1\text{O}_2$ necessary to induce cell death is in reasonable agreement with the one obtained for PDT experiments (see ref. 43 for details).

4.3.3. *Toward New Tools to Study Oxidative Stress*

Motivated by a potentially less-invasive and less-expensive therapy and by the possibility of a simplified generation of singlet oxygen in cells, PS-free production of singlet oxygen also opens up new outlooks on traditional PDT that is intrinsically a complex multiscale and multipartner dynamical system. Predicting therapeutic outcome in PDT requires, among others, knowledge of the amount of cytotoxic species generated. In this context, accurate dosimetry is necessary to ensure complete treatment with consistent and reproducible results for patients. When the singlet oxygen is generated with a PS, dosimetry involves a complex set of interactions including the dynamic of PS, light, oxygen and of the different targets found in the biological tissues. One can emphasize different mechanisms, such as photobleaching of the PS, or the bleaching of its triplet state by $^1\text{O}_2$ setting a nontrivial relationship between the laser light and production of $^1\text{O}_2$.^{45,46} With the direct generation of $^1\text{O}_2$, the definition of the dose is directly related with the fluence of light at 1270 nm and with the intracellular concentration of dioxygen.

The lifetime of $^1\text{O}_2$ is about 3 μs in water, if one considers the case of free diffusion; $^1\text{O}_2$ molecules travel about 100 nm over their lifetime. The spatial distribution of PS thus defines precisely where $^1\text{O}_2$ is generated and it defines a spatial cellular response to oxidative stress.⁴⁷ It should also be noted that the location of PS in specific cellular compartments (mitochondria, the Golgi apparatus, the nucleus, or the plasma membrane) depends on the nature of the PS itself and the cellular types.^{48,49} This localization of the PS can also evolve over time during and after the incubation.

The PS-free laser excitation of molecular dioxygen may help to understand the mechanisms by which PDT is a dynamic system with several partners. It may be noted that the direct excitation of $^1\text{O}_2$ could also provide a reference for studies in PDT in order to discriminate the type-I reactions (radical) and type-II reactions ($^1\text{O}_2$) in response to cellular stress. Being able to overcome the localization of PS and thus the site of intracellular reactive oxygen species (ROS) production allows identifying the most sensitive to the $^1\text{O}_2$ generation cellular compartments and it would help for the design of PS vectorization in PDT. For example, cells deficient in mitochondrial respiration may be less sensitive in PDT. In recent studies, it was shown that the mitochondria could be a source of ROS low dose PDT.⁵⁰ Recently, it was also observed that the

endoplasmic reticulum and lysosomes are also involved in the induction of ROS when cells are photosensitized.⁵¹ It should also be noticed that a precise temporal control of the production of singlet oxygen opens up promising studies in the kinetics of the redox homeostasis at the cellular level.⁸

4.3.4. Toward New Phototherapies?

Due to its simplicity, the PS-free production of singlet oxygen can overcome problems inherent to PDT, like for instance the biodistribution of the PS within the body and its low specificity/selectivity to cancer types, the photosensitivity of the patient and the cost of the treatment. In order to initiate cell death in PS-free photoactivation of singlet oxygen, one has to balance the weak absorption at 1270 nm with a high-power laser leading, for instance, *in vitro* to fluences of *ca.* 100 W cm⁻² h to achieve cell death.⁴³ For PS-free phototherapies, one should notice that strong absorption in water leads to important heating process during 1270 nm laser irradiation. This implies that *in vivo*, thermal stress should also be taken into account to characterize cell death. For instance, these thermal effects could trigger possible necrotic cell death leading to tissue inflammation. The laser-induced temperature increase should be minimized *in vivo* considering production of singlet oxygen at 765 nm (see Section 4.2). Finally, one will have to understand the combination of both oxidative and thermal stress in order to propose new PS-free phototherapies in oncology.⁵²

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Chapter 5

Photosensitization

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5.1. Introduction

In a photosensitized reaction, light energy is absorbed by one chemical species (called the photosensitizer) elevating the photosensitizer to an electronically excited state and ultimately causing a chemical change in a second species (called the substrate or acceptor). Energy transfer from electronically excited photosensitizers to ground-state oxygen is the most common mechanism for generating singlet oxygen. This topic has been reviewed a number of times.¹⁻⁹ Examples of photosensitized singlet oxygen generation can be found both in nature and as a result of human activity. In plants, singlet oxygen is a toxic side product of photosynthesis.¹⁰ Several fungal species and Saint John's wort produce the potent singlet oxygen-generating phototoxins, cercosporin and hypericin, respectively.^{11,12} Many synthetic singlet oxygen-generating photosensitizers have been made in the laboratory. Potential uses of these photosensitizers include synthesis of fine chemicals, photoactivated herbicides, photoactivated insecticides, photodynamic therapy of cancer, photosterilization of blood products and wastewater treatment.¹³⁻¹⁹

5.2. History

In 1867, Fritzsche observed that orange-colored solutions of tetracene were bleached when exposed to light.²⁰ While the mechanism for this process was unknown at the time, this was probably the first description of a photosensitized singlet oxygen-mediated reaction.^{13,20} Roughly three decades later, Oscar Raab and Hermann von Tappeiner found that the toxicity of acridine on paramecia was light dependent.^{21,22} It was later shown that there was an oxygen dependence to this phenomenon.²³ This was probably the first detailed description of a system in which the photochemical generation of singlet oxygen produced biological damage. However, the mechanism responsible for the killing of the paramecia remained unknown.

In the 1930s, Kautsky and coworkers hypothesized that many photosensitized oxidations were mediated by singlet oxygen, with the singlet oxygen being generated by energy transfer from excited photosensitizers.^{24,25} Their singlet oxygen hypothesis was based on experiments in which a photosensitizer, separated from a substrate by a gap, still produced a chemical change in the substrate. These investigators concluded that the observed oxidation products were due to a gas species that was able to diffuse across the gap. Initially, this work did not receive much acceptance. However, substantial evidence supporting Kautsky's hypothesis did come roughly 30 years later. In 1964, Foote and Wexler found that the oxidation products in a number of photosensitized reactions were the same as the oxidation products using singlet oxygen as an oxidant.^{26,27} These investigators used the reaction of hydrogen peroxide with hypochlorous acid to generate the singlet oxygen. Independently, Corey and Taylor found that the oxidation products from other photosensitized reactions were the same as the oxidation products produced by singlet oxygen.²⁸ Corey and Taylor used a radio-frequency discharge in oxygen to produce the singlet

oxygen. Following these landmark studies, the photosensitized production of singlet oxygen became a well-accepted mechanism for photo-oxidation.

5.3. Photosensitizers

5.3.1. Laws of Photochemistry

In the early nineteenth century, it was recognized that only light absorbed by a system can cause a chemical change in that system. This is called the Grotthus–Draper law or first law of photochemistry. A more recent formulation of this principle is the Stark–Einstein law or second law of photochemistry. This law states that the number of activated molecules is equal to the number of photons absorbed.

Two-photon excitation of photosensitizers is an exception to the Stark–Einstein law.²⁹ This phenomenon requires extreme light intensities that are usually generated only with pulsed lasers. The two photons must interact with the photosensitizer within a femtosecond time period. This is roughly the time required for an electronic transition to occur. Absorption of the first photon is felt to produce a “virtual state”. The final excitation energy of the photosensitizer will be equal to the sum of the energy of the two photons absorbed.

5.3.2. Electronic Energy States and Transitions in Isolated Photosensitizers

Figure 5.1 shows the energy states for a prototypical photosensitizer.³⁰ Singlet states are denoted by the letter S and triplet states by the letter T. The ground state is denoted S_0 . Note that the T_1 state is the lowest excited state. Also, note

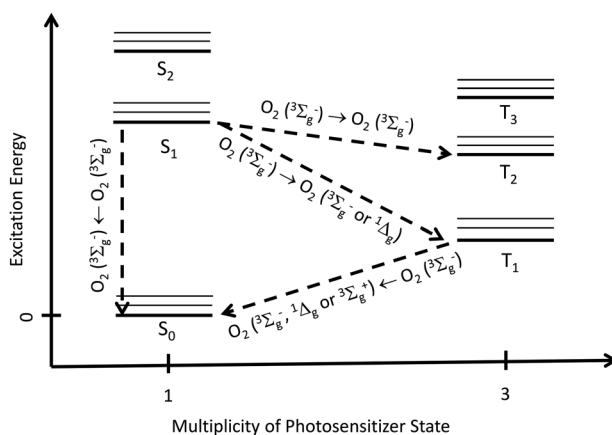


Figure 5.1. Energy states for a prototypical photosensitizer. Singlet states are denoted by S; triplet states are denoted by T. Selected oxygen-mediated transitions are also shown. The transitions shown are all spin allowed. These transitions are also exothermic for some photosensitizers. Transitions, that are energetically unfavorable or for which there is little experimental evidence are not shown.

that the S_1 excited state is above the S_0 ground state, the T_1 state and possibly other T states. Absorption of a photon initially puts the photosensitizer into an excited S state. The photosensitizer may then lose all or part of its energy by dropping into a lower energy S state. If a photon of light is released, this process is called fluorescence.³¹ If there is no photon released and the energy is ultimately dissipated as heat, the process is called internal conversion. Alternatively, the photosensitizer may undergo an intersystem crossing to a T state having a lower excitation energy. Since transitions between excited triplet states are allowed, photosensitizer molecules in higher triplet states usually drop down quickly to the T_1 state. Since transitions from excited triplet states to the singlet ground state are spin forbidden, the lowest-energy triplet state tends to be long lived. However, transitions from a T state to the ground state with release of a photon do occur on occasions. This process is called phosphorescence.³¹

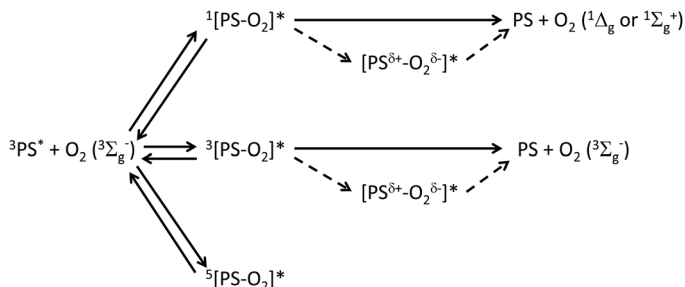
5.3.3. *Oxygen Quenching of Excited Photosensitizers*

Oxygen is known to quench both fluorescence and phosphorescence. This shows that oxygen can quench excited photosensitizers in both singlet and triplet states.³¹ Under the right circumstances, quenching of singlet states or of triplet states can produce singlet oxygen.

5.3.3.1. *Oxygen Quenching of Excited Photosensitizers in Triplet States.* Triplet-state quenching is discussed first because it is the most common mechanism producing singlet oxygen. The process is also easier to analyze than singlet-state quenching, because there is only one photosensitizer transition possible. This transition is to the ground state. The lowest triplet state is often relatively long lived and thus is quenched by relatively low concentrations of oxygen. This is true in spite of the fact that the quenching rates in solution are, generally, at least one order of magnitude slower than the diffusion-controlled rate.^{5,6}

Scheme 5.1 outlines the mechanisms for quenching of the photosensitizer T_1 state. The first step is the formation of an encounter complex from a collision between an oxygen molecule and an excited photosensitizer molecule.^{5,6} The complex may decompose releasing the photosensitizer molecule and the oxygen molecule, both unchanged. Alternatively, the complex may proceed along a reactive channel to yield products. The encounter complex can have a multiplicity of 1, 3 or 5. The products of the singlet complex are a ground-state photosensitizer molecule and a molecule of singlet oxygen (either $^1\Delta_g$ or $^1\Sigma_g^+$). This is a spin-allowed process. The products of the triplet complex are a ground-state photosensitizer molecule and a ground-state oxygen molecule. This is also spin allowed. There are no reactive channels for the quintet complex.

For some photosensitizers, particularly those with high oxidation potentials, the principal mechanism for quenching is internal conversion of the encounter complex. For the reaction to be exothermic, the excitation energy



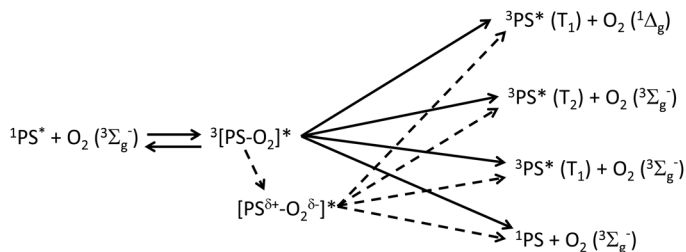
Scheme 5.1. Mechanisms for quenching of excited photosensitizer triplet states by oxygen. Here, $^3\text{PS}^*$ represents a photosensitizer molecule in the lowest excited triplet state, PS represents a photosensitizer molecule in the ground state, $[\text{PS}-\text{O}_2]^*$ represents an encounter complex and $[\text{PS}^{\delta+}-\text{O}_2^{\delta-}]^*$ represents a charge-transfer exciplex.

of the complex must be above the sum of the excitation energies of the final products. For oxygen in the $^1\Delta_g$ state, this is 94 kJ mol^{-1} . For oxygen in the $^1\Sigma_g^+$ this is 157 kJ mol^{-1} . For ground-state oxygen and for ground-state photosensitizer, there is no excitation energy. In general, some additional excitation energy will need to be dissipated by the complex as it undergoes internal conversion. As a general rule, the greater the energy dissipation required, the slower the quenching rate.³²

For photosensitizers with low oxidation potentials, an additional reactive channel is available. The collision complex can be stabilized by charge transfer from the photosensitizer to oxygen, thus forming an exciplex and reducing the amount of excess excitation energy that ultimately needs to be dissipated. Consequently, photosensitizers with low oxidation potentials tend to be quenched more quickly than photosensitizers with high oxidation potentials.³³

5.3.3.2. Oxygen Quenching of Excited Photosensitizers in Singlet States. In solution, oxygen generally quenches excited singlet-state photosensitizers at near diffusion-controlled rates.^{5,6} However, the lifetimes of the excited singlet states are so short that efficient quenching by oxygen usually requires relatively high oxygen concentrations. In addition, the singlet oxygen quantum yield is generally less than one at moderate oxygen concentrations because photosensitizer quenching by oxygen always competes with fast oxygen-independent deactivation processes.

Scheme 5.2 outlines the mechanism for oxygen quenching of excited singlet states. The first step is the formation of an encounter complex.^{5,6} The encounter complex has triplet multiplicity. In some cases the encounter complex will lead to the formation of a charge-transfer exciplex, which can then undergo internal conversion, ultimately forming products. Four different pairs of products have been reported from excited singlet-state quenching. All of the reported pairs of products are the result of spin-allowed transitions.



Scheme 5.2. Mechanisms for quenching of excited photosensitizer singlet states by oxygen. Here, $^1\text{PS}^*$ represents a photosensitizer molecule in the lowest excited singlet state, $^3\text{PS}^*$ represents a photosensitizer molecule in an excited triplet state, ^1PS represents a photosensitizer molecule in the ground state, $[\text{PS}-\text{O}_2]^*$ represents an encounter complexes and $[\text{PS}^{\delta+}-\text{O}_2^{\delta-}]^*$ represents a charge-transfer exciplex.

The first pair of products is composed of a $^1\Delta_g$ oxygen molecule and T_1 photosensitizer molecule. The energy difference between the S_1 and T_1 states of photosensitizers is generally not large enough to elevate oxygen to the $^1\Sigma_g^+$ state.^{5,6} Production of both $^1\Delta_g$ oxygen and T_1 photosensitizer is of particular interest because it implies that the overall singlet oxygen quantum yield can be as high as 2. This is because the T_1 photosensitizer molecule that is produced can subsequently be quenched by another oxygen molecule to produce a second molecule of singlet oxygen. For this process to be exothermic, the S_1 energy state must be at least 94 kJ mol^{-1} above the T_1 energy state. Indeed, there is good evidence that this is true for rubrene and for a few other photosensitizers.^{34–37}

The second possible pair of products shown in Scheme 5.2 is ground-state oxygen and photosensitizer in the T_2 state. For a few photosensitizers, this mechanism has been shown to enhance the overall photosensitizer triplet yield.^{36,37} The third pair of products in Scheme 5.2 is ground-state oxygen and T_1 photosensitizer. This reaction channel is significant for many photosensitizers when there is insufficient excitation energy to produce singlet oxygen. The fourth pair of products is ground-state oxygen and ground-state photosensitizer.³⁸ In contrast to the first three pairs of products, the mechanism for the fourth pair does not involve intersystem crossing. Finally, this fourth mechanism seems to occur efficiently in only a few photosensitizers.

5.4. Photochemical Mechanisms Competing with Singlet Oxygen Formation

Photosensitized singlet oxygen generation always competes with dissipation of the absorbed light energy as heat. In fact, most light-absorbing molecules are poor photosensitizers because most of the absorbed energy is ultimately dissipated as heat. This can occur as a result of radiationless decay to the ground state (internal conversion) or enhanced internal conversion as a

result of collisions with other molecules. Photosensitized singlet oxygen generation must also compete with other mechanisms for photosensitization. Alternative mechanisms for photosensitization include charge transfer to oxygen, charge transfer to other molecules and energy transfer to molecules other than oxygen.

As shown in eqn (5.1), an excited photosensitizer can transfer an electron to oxygen producing a positively charged photosensitizer and superoxide anion.³⁹ However, the quantum yields for superoxide production are usually low. In some systems, a subsequent reaction between the oxidized photosensitizer and a reducing agent can regenerate the photosensitizer.^{40,41} This is shown in eqn (5.2), where R represents the reducing agent.



Excited photosensitizers can also transfer charge to molecules other than oxygen. More often the photosensitizer is reduced rather than oxidized. This is shown in eqn (5.3).



An example of this mechanism is the photoreduction of chlorophyll.⁴²

Finally, photosensitizers can transfer energy to molecules other than oxygen. It has been suggested, but not well documented, that energy transfer to biomolecules might be a mechanism responsible for some phototoxic reactions.⁴³ The major weakness with this hypothesis is that most biomolecules do not have sufficiently low-lying energy states to accept energy from commonly used photosensitizers.⁴⁴ In contrast to phototoxicity, energy transfer to biomolecules is an established mechanism for photoprotection.^{45,46} Carotenoids have low-energy excited states and can quench both excited photosensitizers and singlet oxygen.^{45,46} Most often, the energy transferred to carotenoids is ultimately dissipated as heat.

5.5. Classification Schemes for Photosensitized Reactions

Photosensitized reactions often have complex mechanisms with an initial light-requiring elementary reaction, followed by several light-independent secondary reactions (*e.g.*, radical chain reactions). Consequently, there had been considerable interest in having a simple classification scheme. Many authors have classified photosensitized reactions into two broad categories, called Type I and Type II.⁴⁷ Initially, the distinction between Type-I photochemistry and Type-II photochemistry depended on the nature of the intermediates produced.⁴⁷ Type-I photochemistry proceeded through monoradical intermediates and Type-II photochemistry proceeded “diradicals” intermediates. It was later established that the “diradicals” were in fact singlet oxygen molecules.

The concept of Type-I and Type-II photosensitized reactions was refined by Foote.⁴⁸ He proposed that an experimental, rather than a mechanistic classification, was most appropriate. If the excited photosensitizer initially reacted with a substrate or solvent, the reaction was Type I. If the excited photosensitizer initially reacted with oxygen, the reaction was a Type II. Type-II reactions included those involving electron transfer from the photosensitizer to oxygen, thus producing superoxide anion. Foote favored these definitions because he thought that it was relatively easy experimentally to determine the initial reactant for an excited photosensitizer.

An opposing view was given by Vidòczy.⁴⁹ He proposed that Type-I reactions should be defined as charge transfer from the photosensitizer to another molecule. Type-II reactions were defined as energy transfer from the photosensitizer to another molecule. Vidòczy thought that these definitions provided a more general classification of photosensitized reactions and that in some circumstances (*e.g.*, complex biological systems), it might be very difficult to determine the initial reactant for a photosensitizer.

More recently Krasnovsky has emphasized utility of classifying photosensitized reactions based on the primary photoreaction and how this reaction activates oxygen.⁸ He suggested that oxygen activation as a result of electron-transfer reactions should be classified as Type I and that oxygen activation as a result of energy transfer should be classified as Type II. A classification scheme based on the nature of subsequent intermediates is less useful because secondary reactions almost always involve both free radicals and singlet oxygen. Indeed singlet oxygen is often a secondary product from the reactions of free radicals. The Russell mechanism for the recombination of two peroxy radicals is one example of secondary singlet oxygen production.^{50,51}

The mechanisms of many photosensitized reactions are now known in extreme detail. This diminishes the usefulness of classifying reactions as Type I or Type II. This is particularly true given the long-standing arguments about the definitions of Type-I and Type-II reactions. It may be best to classify primary photosensitized reactions as involving charge transfer or energy transfer and to specify the acceptor molecule (oxygen or other molecule) involved.

5.6. Factors Favoring Singlet Oxygen Formation over Other Photosensitized Mechanisms

Four factors are responsible for the fact that singlet oxygen generation is often the dominant mechanism of photosensitization.⁶ First, oxygen molecules have an unusual electronic structure with two low-lying singlet states. Secondly, the lowest triplet state of many photosensitizers is sufficiently energetic to excite oxygen to one of its singlet states in a spin-allowed transition. Thirdly, oxygen is a small molecule with a large diffusion coefficient. Finally, oxygen is present at a high concentration in the environment.

Efficient singlet oxygen generation also requires a good photosensitizer. The properties of good photosensitizers include a high extinction coefficient at the desired wavelength of light, a high quantum yield for production of a triplet state, a long lifetime for the triplet state, sufficient excitation energy in the triplet state to populate the $^1\Delta_g$ state of oxygen and resistance to oxidation by singlet oxygen and any secondary oxidants present.

5.7. Conclusions

The photosensitized production of singlet oxygen is quite common. The novel electronic structure of oxygen and its high concentration in the environment often makes singlet oxygen generation a dominant photochemical pathway. Much effort has been devoted to develop a detailed understanding of the photochemical mechanisms that result in singlet oxygen generation and to develop practical applications of this photochemistry.

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Chapter 6

Reference Photosensitizers for the Production of Singlet Oxygen

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6.1. Introduction

The number of singlet oxygen molecules produced per amount of photons absorbed by a photosensitizer (per unit of time), *i.e.*, the quantum yield of photosensitized singlet oxygen production (Φ_{Δ}) is a parameter of major importance for the optimized development of specific applications using singlet oxygen. Therefore, Φ_{Δ} has to be accurately known for any photosensitizer under the experimental conditions used if quantitative results have to be obtained. Most of the laboratory methods currently used for the determination of the absolute Φ_{Δ} value of any novel sensitizer, or that of an already known photosensitizer under distinct experimental conditions (*e.g.*, techniques based on photo-oxidation reactions using singlet oxygen acceptors, or detection of the $^1\text{O}_2$ phosphorescence in the near-IR region, as exemplified in other chapters of this book) are relative methods. They are based on comparisons of experimental results obtained under the same conditions for the sample (sensitizer of unknown Φ_{Δ}) and for standard or reference sensitizers of Φ_{Δ} values that are known with high enough precision (% relative error $\leq \pm 10\%$). Therefore, there is a need for efficient singlet oxygen photosensitizers for which Φ_{Δ} values in different media have been thoroughly determined, preferably in more than one laboratory, with consistent results of minimal uncertainty. Such photosensitizers could be considered as established standards for singlet oxygen production.

6.2. Singlet Oxygen Photosensitizers and Factors Affecting Their Efficiency

Among the chemical substances that generate $^1\text{O}_2$ upon irradiation with light of suitable wavelength, almost all the families of dyes can be found: polynuclear aromatic hydrocarbons, aromatic ketones, thiones, quinones; heterocycles such as coumarins, fluorescein and its polyhalogenated derivatives (*e.g.*, rose bengal), acridines, phenazines and phenothiazines (*e.g.*, methylene blue); carbon nanoforms such as fullerenes (C_{60} and its mono-, bis-, tris-, tetra- and hexa-adducts, and larger fullerenes like C_{70} , C_{76} , C_{84}) and single-walled carbon nanotubes; porphyrins, phthalocyanines and several of their metal complexes, and also the coordination compounds of many transition metals, mainly Ru(II), with polyazaheterocyclic ligands like 2,2'-bipyridine, 1,10-phenanthroline and their derivatives (Figure 6.1). The extensive review by Wilkinson, Helman and Ross in 1993 thoroughly describes the methods for studying the kinetics and reactions of singlet oxygen, and compiles Φ_{Δ} values for a great many photosensitizers.¹ This compilation of data was further extended by Redmond and Gamlin in 1999.² On the other hand, the excellent review by Schweitzer and Schmidt in 2003 discusses in detail the physical mechanisms of $^1\text{O}_2$ generation and deactivation, including those parameters influencing the photosensitized $^1\text{O}_2$ production.³

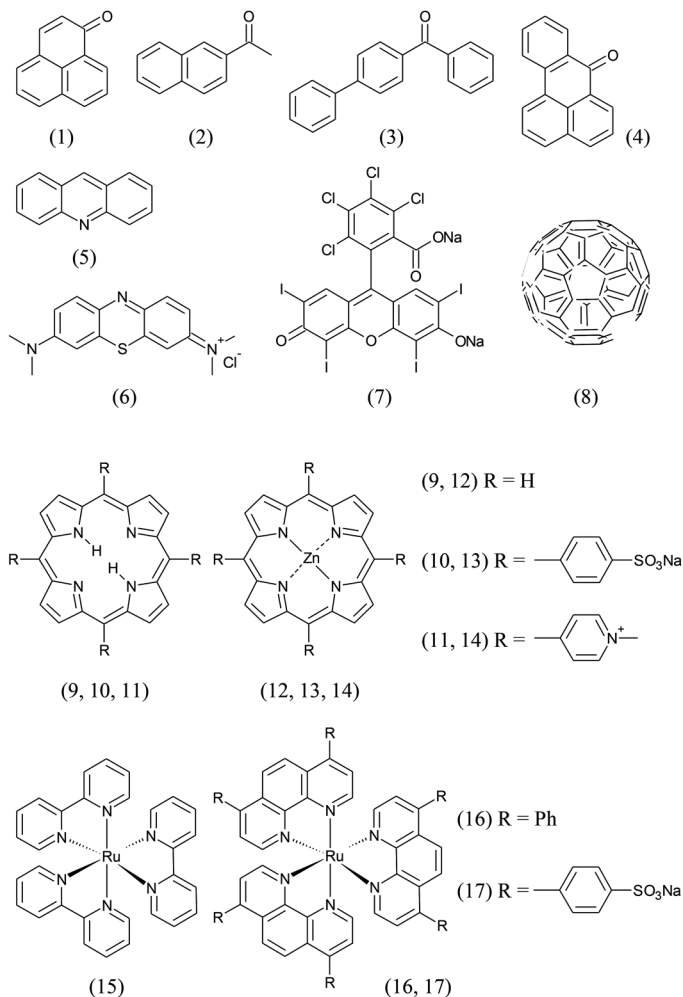


Figure 6.1. Structures of standard photosensitizers for singlet oxygen production.

In general, Φ_{Δ} can be expressed by the sum of two contributions due to quenching of $^1\text{PS}^*$ and/or $^3\text{PS}^*$ by O_2 (eqn (6.1)):

$$\Phi_{\Delta} = P_{\text{S}}^{\text{O}_2} \times f_{\text{S},\Delta}^{\text{O}_2} + \Phi_{\text{T}}^{\text{O}_2} \times P_{\text{T}}^{\text{O}_2} \times f_{\text{T},\Delta}^{\text{O}_2}, \quad (6.1)$$

where $P_{\text{S}}^{\text{O}_2}$ stands for the proportion of singlet states quenched by O_2 , $f_{\text{S},\Delta}^{\text{O}_2}$ represents the fraction of those singlet states quenched by O_2 that produce singlet oxygen, $\Phi_{\text{T}}^{\text{O}_2}$ is the quantum yield of triplet formation in the presence of O_2 , $P_{\text{T}}^{\text{O}_2}$ accounts for the probability of triplet-state quenching by O_2 , and $f_{\text{T},\Delta}^{\text{O}_2}$ stands for the fraction of those triplet states quenched by O_2 that give singlet oxygen.

From eqn (6.1), it can be inferred that the Φ_{Δ} value of a photosensitizer in a given medium is strongly dependent on a series of variables such as (i) the proportion of excited states quenched by O_2 ($P_S^{O_2}$ and/or $P_T^{O_2}$), (ii) the fraction of excited states quenched by O_2 that leads to $^1O_2$ production ($f_{\Delta}^{O_2} = k_{en}^{O_2}/k_q^{O_2}$, where $k_{en}^{O_2}$ is the rate constant of energy transfer from $^1PS^*$ and/or $^3PS^*$ to O_2 and $k_q^{O_2}$ is the overall quenching rate constant by oxygen) and (iii) the quantum yield of formation of the sensitizer triplet state (Φ_T , due to either enhanced triplet formation from the excited singlet in the presence of O_2 ($\Phi_T^{O_2}$), or direct intersystem crossing (Φ_{ISC}) from the excited singlet to triplet state). All these variables are related to intrinsic and extrinsic factors depending on the molecular structure of the photosensitizing dye (photophysical properties) and also on the nature of the surrounding medium (microenvironment, *i.e.*, solvent, oxygen concentration, temperature and pressure), respectively. In the case of the intrinsic factors, these variables may be correlated with several sensitizer features or parameters that can be experimentally determined, such as the type and energy of the excited state, its lifetime, its quenching constant by O_2 and its oxidation potential. However, it has to be taken into account that parameters such as $f_{\Delta}^{O_2}$, $k_q^{O_2}$ and P^{O_2} (regardless of the singlet or triplet character of the excited state) are also strongly dependent on extrinsic factors such as oxygen concentration (*e.g.*, P^{O_2}) and the nature of the solvent – mainly its polarity and viscosity – (as is the case for $f_{\Delta}^{O_2}$ and $k_q^{O_2}$). Values of Φ_{Δ} become independent of the O_2 concentration when the latter is higher than 2×10^{-4} M, only for compounds with long-lived triplet states ($P_T^{O_2} \geq 0.95$ if τ_T^0 is longer than *ca.* 15–20 μs for air-equilibrated solutions) and Φ_{ISC} independent of the O_2 concentration. In this sense, it has to be taken into account that aqueous solutions are a special case since oxygen solubility in air-equilibrated water is slightly higher than 0.25 mM, about one order of magnitude lower than for air-saturated common organic solvents.

Because of their variety and their complexity, the parameters determining the efficiency of photosensitized singlet oxygen production should be discussed in more detail.

6.2.1. Nature and Relative Energy of the Lowest Excited State of the Photosensitizer

According to the nature of the excited state quenched by O_2 (S_1 or T_1), and whether the energy difference ($E_{S1} - E_{T1}$) is larger or smaller than E_{Δ} , a classification of singlet oxygen sensitizers into three categories abbreviated as ST, TC and T type sensitizers was proposed:¹

6.2.1.1. ST Photosensitizers. The term ST is used for those photosensitizers with $(E_{S1} - E_{T1}) > E_{\Delta}$ and $E_{T1} > E_{\Delta}$, which are able to produce singlet oxygen from their lowest excited singlet and also triplet states [*via* the spin-allowed $^1PS^* + O_2(^3\Sigma_g^-) \rightarrow ^3PS^* + O_2(^1\Delta_g)$ and $^3PS^* + O_2(^3\Sigma_g^-) \rightarrow PS + O_2(^1\Delta_g)$]

deactivation processes]. These sensitizers may have a limiting Φ_{Δ} value of 2 (all terms in eqn (6.1) equal to 1), due to the possibility of singlet oxygen production from their excited singlet state and, subsequently, after intersystem crossing, from their triplet state (*e.g.*, many polynuclear aromatic hydrocarbons and their derivatives, such as rubrene, heterococordinthron, tetracene, perylene, pyrene, chrysene, and many anthracenes).³ It should be noted, however, that, for these ST sensitizers also, Φ_{Δ} values measured experimentally may be much lower than 1. A special behavior could be found for some photosensitizers lying in this category for which $f_{s,\Delta}^{O_2} = 0$, even though $(E_{S1} - E_{T1}) > E_{\Delta}$. In this case, sensitization of $^3PS_2^*$ from $^1PS^*$ is possible (*via* O_2 -enhanced ISC in encounter complexes of triplet multiplicity) since $^3PS_2^*$ lies slightly below or above $^1PS^*$ ($^3PS_2^*$ can be thermally accessible in the latter case). In this specific situation, efficient deactivation can occur *via* rapid $^3PS_2^* \rightarrow ^3PS^*$ internal conversion yielding the sensitizer in its ground state without $^1O_2$ production.^{4,5}

6.2.1.2. TC Photosensitizers. The term TC refers to those photosensitizers with $(E_{S1} - E_{T1}) < E_{\Delta}$ but $E_{T1} > E_{\Delta}$ (*e.g.*, fluoranthenes)^{3,6} that cannot produce singlet oxygen from their excited singlet state ($f_{s,\Delta}^{O_2} = 0$) but have the possibility to catalyze the intersystem crossing by O_2 quenching of the singlet state ($P_s^{O_2} \neq 0$ and $\Phi_T^{O_2} \neq 0$) producing the triplet state *via* the $^1PS^* + O_2(^3\Sigma_g^-) \rightarrow ^3PS^* + O_2(^3\Sigma_g^-)$ pathway. Therefore, for TC sensitizers, singlet oxygen can be generated more or less effectively depending on the value of $P_s^{O_2}$ that rules the triplet yield of the photosensitizer. Since, in general, all the O_2 quenching pathways compete with relatively efficient intramolecular deactivation processes (*e.g.*, fluorescence emission or internal conversion), low $P_s^{O_2}$ values are typically found in air-equilibrated solutions. Most of the sensitizers displaying long enough $^1PS^*$ lifetimes (tens of ns) lie in this category, where the role of O_2 quenching of $^1PS^*$ is limited to enhancement of the triplet quantum yield.

6.2.1.3. T Photosensitizers. This third group refers to those photosensitizers that can produce singlet oxygen only from their excited triplet state due to highly efficient intersystem crossing ($\Phi_{ISC} \approx 1$, $P_s^{O_2} = 0$) and concomitant spin-allowed energy transfer from $^3PS^*$ to ground-state molecular oxygen (a triplet state: $^3\Sigma_g^-$). These photosensitizers can have a maximum Φ_{Δ} value of 1 (*e.g.*, 1*H*-phenalen-1-one).⁷

6.2.2. Photosensitizer Triplet Quantum Yield

A high triplet quantum yield (Φ_T) is a common feature of any useful singlet oxygen sensitizer regardless of its belonging to ST, TC or T classification type. $^3PS^*$ production in the presence of O_2 ($\Phi_T^{O_2}$) in ST- and TC-type photosensitizers, as discussed above, is due to quenching of relatively long-lived $^1PS^*$

by O_2 resulting in spin-allowed $^3PS^*$ formation that, in turn, produces $^1O_2$. On the other hand, T-type photosensitizers undergo intersystem crossing from $^1PS^*$ to $^3PS^*$ (Φ_{ISC}) with high efficiency (typical of sensitizers containing heavy atoms or aromatic molecules with high electron delocalization, such as polynuclear aromatic hydrocarbons or aromatic ketones with strong spin-orbit coupling). If $^3PS^*$ is sufficiently long lived (tens of μs), quantitative quenching by O_2 may occur even in air-equilibrated solutions and Φ_Δ will only depend on the $f_{T,\Delta}^{O_2}$ value for T-type photosensitizers with high Φ_{ISC} .

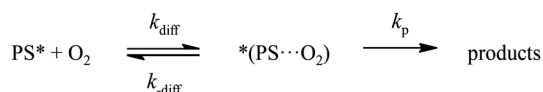
Very recently, the effect of heavy-atom insertion on the ISC efficiency through spin-orbit perturbations of a new class of picolylamine-porphyrin conjugates has been investigated. By incorporating Zn(II) ions in the core as well as at the periphery of the porphyrin ring, Φ_{ISC} and Φ_Δ values have been successfully optimized. A picolylamine-porphyrin conjugate having five Zn(II) ions exhibited a Φ_{ISC} of 0.97 and a Φ_Δ of 0.92. In contrast, the free base porphyrin derivative exhibited a Φ_{ISC} of 0.64 and Φ_Δ of 0.5 only.⁸

Many types of photosensitizers show a strong Φ_T dependence on the solvent nature (solvent polarity and protic character) because of the solvent-induced changes in the relative energies of $^1PS^*$ and $^3PS^*$, and even in the nature ($\pi\pi^*$ or $n\pi^*$) of the excited states with lowest energy, or by modifying the deactivation rates of the excited states (*e.g.*, by promoting internal conversion). The effect of the solvent features on the sensitizer triplet quantum yield will be discussed in Section 6.2.7.

6.2.3. Photosensitizer Excited-State Lifetime and Rate Constant of Bimolecular Quenching by Molecular Oxygen

Quenching by molecular oxygen can only occur when the lifetime of the excited state (τ^0) is long enough for the excited sensitizer to encounter an oxygen molecule. The phenomenological approach to quenching kinetics involves the formation of a collision complex between the excited sensitizer (PS^*) and O_2 from which the final products or excited states are formed, according to Scheme 6.1.

Diffusional encounter of PS^* and O_2 molecules (proceeding with a second-order rate constant k_{diff}) produces the collision complex $^*(PS\cdots O_2)$ within a solvent cage. This excited complex lives for less than a nanosecond because it can be deactivated by two competitive pathways: products formation (with a first-order rate constant k_p) or break up of the solvent cage and release of PS^* and O_2 into the bulk solvent (with a first-order rate constant k_{-diff}). Applying the steady-state approximation for the concentration of $^*(PS\cdots O_2)$, the



Scheme 6.1. Description of the bimolecular quenching process of an excited sensitizer by molecular oxygen.

observed quenching rate constant $k_q^{O_2}$ for the bimolecular deactivation of PS* by O_2 can be expressed by eqn (6.2):

$$k_q^{O_2} = \frac{k_{diff} \times k_p}{k_{diff} + k_p}, \quad (6.2)$$

where k_{diff} ($M^{-1} s^{-1}$) can be calculated using Smoluchowski's theory for diffusion-controlled reactions. When product formation is much faster than the break-up of the collision complex into the original species ($k_p \gg k_{diff}$), $k_q^{O_2}$ in eqn (6.2) reduces to the diffusion-controlled rate constant (k_{diff}). In this case, Smoluchowski's equation for the calculation of the rate constant for an irreversible bimolecular diffusion-controlled reaction allows for a theoretical estimation of $k_q^{O_2}$ ($k_{Smoluchowski}$) as:

$$k_{Smoluchowski} = 4\pi \times \frac{R^* \times D \times N_A}{1000}, \quad (6.3)$$

where R^* , D , and N_A denote the reaction distance (the collision radius, $R^* = R_{PS^*} + R_{O_2}$), the mutual diffusion coefficient of the reactants ($D = D_{PS^*} + D_{O_2}$), and the Avogadro number (N_A), respectively. The diffusion coefficients of the reactants ($m^2 s^{-1}$) may be obtained from empirical equations, such as the Stokes–Einstein relation:

$$D_{S-E} = \frac{k_B \times T}{6\pi \times \eta \times R}, \quad (6.4)$$

where k_B , T , η and R denote the Boltzmann constant ($J K^{-1}$), the absolute temperature (K), the solvent viscosity (Pa s) and the radius of the solute (m), respectively.

Smoluchowski and Stokes–Einstein equations clearly evidence the strong inverse dependence of $k_q^{O_2}$ on the solvent viscosity. However, in the particular case of O_2 , which is a small molecule compared with other solutes (*e.g.*, sensitizers) and diffuses much faster through the solvent, the effect of viscosity on $k_q^{O_2}$ is smaller than expected by theoretical calculations.³ Typical viscosities of common organic solvents (nonpolar, polar aprotic or polar protic) are between 0.2 and 2.0 mPa s at 25 °C, in the low-viscosity range, and the estimated diffusion-controlled rate constants for a generic dye and quencher in common organic solvents will be between 3.2×10^9 and $31 \times 10^9 M^{-1} s^{-1}$.⁹ Similarly, an estimation of the diffusion-controlled rate constant in aqueous phase from Smoluchowski's theory using water viscosity data (0.89 mPa s, 25 °C) gives a $k_{Smoluchowski}$ of $7.4 \times 10^9 M^{-1} s^{-1}$. On the other hand, in the case of a highly viscous medium such as glycerol (945 mPa s, 25 °C) a $k_{Smoluchowski}$ of $7.0 \times 10^6 M^{-1} s^{-1}$ can be estimated.⁹

The lifetime of the photosensitizer excited state (τ^0) and the rate constant of bimolecular quenching by molecular oxygen ($k_q^{O_2}$) are experimental parameters easy to determine (*e.g.*, by time-resolved quenching experiments). Both parameters are related to the probability of excited-state

quenching, P^{O_2} (eqn (6.5)), that can be derived from the Stern–Volmer equation for bimolecular dynamic quenching:

$$P^{O_2} = 1 - (I/I^0) = 1 - (\tau/\tau^0) = k_q^{O_2} \times \tau \times [O_2] = \frac{k_q^{O_2} \times [O_2]}{k_d + k_q^{O_2} \times [O_2]}, \quad (6.5)$$

where I and I^0 (for steady-state experiments of emission intensity quenching) or τ and τ^0 (for time-resolved emission quenching or transient absorption experiments) are the emission intensities or lifetimes of the sensitizer excited state in the presence and absence of O_2 , respectively; and k_d is the rate constant of the excited-state deactivation in the absence of oxygen ($k_d = 1/\tau^0$).

In general, reported $k_{s,q}^{O_2}$ are diffusion controlled in condensed phase for most of the sensitizers in their singlet excited state, where $k_{diff} \approx 3 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ is the diffusion-controlled limit for rate constants of reactions with O_2 in common organic solvents at room temperature (e.g., $k_{s,q}^{O_2} = 2.5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ for the anthracene singlet excited state in cyclohexane)⁹ meaning that every encounter between $^1\text{PS}^*$ and O_2 leads to quenching. For triplet excited states, $k_{t,q}^{O_2}$ are often one order of magnitude lower (e.g., $k_{t,q}^{O_2} = 3.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ for anthracene triplet in cyclohexane)⁹ due to a theoretical factor of 1/9 or 4/9, that can be explained, and experimentally evidenced,¹⁰ on the basis of the spin statistics for each possible multiplicity (singlet [1/9], triplet [3/9] or quintet [5/9]) of the excited encounter complex formed by $^3\text{PS}^*$ and $O_2(^3\Sigma_g^-)$ and also on the balance between the competitive deactivation pathways (without charge transfer or with partial charge transfer) in the collision complex.³ Theoretical considerations also predict a dependence of $k_{t,q}^{O_2}$ on the excess of energy of $^3\text{PS}^*$ with respect to that of $^1O_2$, since $k_{t,q}^{O_2}$ values tend to decrease with increasing E_{T1} . Large $k_q^{O_2}$ values ($\approx 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ for singlet excited states, or one order of magnitude lower for triplet excited states) are reflected in very low lifetime values in the presence of O_2 (τ). Therefore, when the sensitizers have long enough excited state lifetimes (τ^0) and large $k_q^{O_2}$ values, high P^{O_2} ($P_s^{O_2}$ or $P_t^{O_2}$) can be achieved, promoting efficient singlet oxygen production (provided that the $f_\Delta^{O_2}$ value, i.e., the ratio $k_{en}^{O_2}/k_q^{O_2}$ is also high).

Eqn (6.5) also reveals the key role played by the oxygen concentration in controlling the value of Φ_Δ , since the lowest possible τ values are generally obtained for O_2 -saturated systems and, therefore, the highest P^{O_2} values can be attained under these conditions.¹¹

6.2.4. Photosensitizer Oxidation Potential

As suggested above, the collision complex formed between the excited sensitizer and O_2 can have some charge-transfer character. Therefore, in competition with the transfer of electronic energy from PS^* to O_2 , leading to $^1O_2$ formation (energy-transfer mechanism), another primary mechanism involving transfer of one electron from PS^* to O_2 , with the simultaneous reduction of O_2 to superoxide anion $O_2^{\cdot-}$ and oxidation of the excited sensitizer to its radical cation ($\text{PS}^{\cdot+}$) may also take place (oxidative electron-transfer

mechanism). Several studies have correlated the parameters $k_q^{O_2}$ and $f_{\Delta}^{O_2}$ with the sensitizer oxidation potential (E_{ox}) when the excited sensitizer can be easily oxidized by O_2 . Therefore, charge transfer (CT) interactions strongly influence the rate and efficiency of $^1O_2$ formation.

For instance, concerning the quenching of singlet excited states by O_2 , small $k_{s,q}^{O_2}$ values have been determined in experiments with $^1PS^*$ sensitizers with high oxidation potentials.^{12–19} Also, $k_{s,q}^{O_2}$ has been observed to decrease with the increasing free energy change of complete electron transfer (ΔG_{CT}),¹⁴ which can be calculated using the Rehm–Weller eqn (6.6)

$$\Delta G_{CT} = F(E_{ox} - E_{red}) - E_{exc} + C, \quad (6.6)$$

where F is the Faraday constant, E_{ox} the ground-state oxidation potential of the sensitizer (determined for the $PS/PS^{+\cdot}$ oxidation half-reaction under standard conditions), E_{red} the reduction potential of O_2 (e.g., $E_{red}(O_2/O_2^{\cdot-}) = -0.78$ V vs. SCE in acetonitrile), E_{exc} is the excited-state energy (in kJ mol^{-1}), and C is the coulombic term.³

Concerning triplet excited states, $k_{T,q}^{O_2}$ decreases as E_{ox} increases, again evidencing the importance of charge-transfer interactions for efficient excited-state quenching by O_2 .^{20,21} On the other hand, the efficiency of $^3PS^*$ formation during quenching of $^1PS^*$ by O_2 (O_2 -enhanced ISC) has been shown to decrease with decreasing E_{ox} values for polynuclear aromatic hydrocarbons. This is due to strong charge-transfer interactions allowing for efficient $^1PS^* \rightarrow PS$ internal conversion in the excited encounter complex $^*[^1PS \cdots O_2(^3\Sigma_g^-)]$ of triplet multiplicity.^{17,22,23}

Singlet oxygen formation parameters such as $f_{s,\Delta}^{O_2}$ and $f_{T,\Delta}^{O_2}$ also show some variation with sensitizer E_{ox} and, in general, an inverse relationship between $k_q^{O_2}$ ($k_{s,q}^{O_2}$ or $k_{T,q}^{O_2}$) and $f_{\Delta}^{O_2}$ ($f_{s,\Delta}^{O_2}$ or $f_{T,\Delta}^{O_2}$) is found for their dependence on the oxidation potential of the sensitizer. Similarly, $f_{T,\Delta}^{O_2}$ values decrease with increasing E_T , since higher values of the sensitizer triplet energy are related to an increase in the ability of the sensitizer to be oxidized (e.g., $f_{T,\Delta}^{O_2}$ values decrease from 1.0 for anthracene, $E_T = 178 \text{ kJ mol}^{-1}$, to 0.25 for triphenylene, $E_T = 280 \text{ kJ mol}^{-1}$).²⁴ Consideration of the corresponding oxidation potentials also demonstrated that $f_{T,\Delta}^{O_2}$ becomes smaller with decreasing ΔG_{CT} due to deactivation through CT-complex states. These assumptions have been corroborated by systematic investigations carried out with a series of naphthalene derivatives displaying strong variations in E_{ox} while all other relevant parameters including E_T were nearly constant.^{25,26} It was found that an increase in E_{ox} results in a systematic increase in $f_{T,\Delta}^{O_2}$ and a decrease in $k_{T,q}^{O_2}$ (i.e., singlet oxygen formation becomes faster but less efficient with decreasing ΔG_{CT}). Similar results were obtained with derivatives of biphenyl,^{20,21,27} fluorene,²⁸ amines,²⁹ Ru(II) complexes,^{30–32} and benzophenone derivatives,³³ where E_T also remains almost constant upon introduction of E_{ox} -modifying substituents. These results were confirmed in nonpolar as well as in polar solvents.^{20,21} Additionally, these investigations demonstrated that the dependence of $k_{T,q}^{O_2}$ on ΔG_{CT} is much weaker than expected for a complete electron transfer; thus, it was concluded that the rate-determining step involves

complexes bearing partial CT-character. Further investigations have demonstrated that photosensitized singlet oxygen generation during quenching of $\pi\pi^*$ triplet states of aromatic molecules by O_2 can be generally described by a mechanism involving the successive formation of excited noncharge-transfer (nCT) encounter complexes and partial charge-transfer (pCT) exciplexes of singlet and triplet multiplicity, following interaction of O_2 with $^3PS^*$.^{3,34} Since a CT channel and a nCT deactivation channel can compete in the quenching of triplet states by O_2 , a parameter was finally proposed that describes the balance between CT and nCT deactivation channels. This parameter, p_{CT} , describes the relative contribution of CT-mediated deactivation and can be easily calculated for a sensitizer of known triplet energy from its quenching rate constant. Thus, p_{CT} quantitatively influences the efficiencies and the rate constants of $O_2(^1\Sigma_g^+)$, $O_2(^1\Delta_g)$ and $O_2(^3\Sigma_g^-)$ formation in the quenching process, and also describes the balance between both deactivation channels without requiring any knowledge of oxidation potentials.³⁵ The p_{CT} parameter can be calculated for different types of photosensitizers such as aromatic hydrocarbons (naphthalene, biphenyl and fluorene derivatives) and Ru(II), Re(I), Os(II) and Ir(III) complexes as well.³⁶

6.2.5. Steric and Structural Effects

Few studies have been focused on the effects of steric changes in the photosensitizer molecular structure on Φ_Δ . Studies carried out mainly with Ru(II) coordination compounds have shown some ligand effects on $k_{T,q}^{O_2}$ for triplet states.^{31,32,37,38} A decrease in $f_{T,\Delta}^{O_2}$ was observed for a series of Ru(II) complexes, in going from the tris-homoleptic 2,2'-bipyridine (bpy) complex $[Ru(bpy)_3]^{2+}$ to the bis-heteroleptic complex $[Ru(bpy)_2L]^{2+}$ and to the bulky tris-homoleptic $[RuL_3]^{2+}$, where L are different 4,4'-disubstituted-2,2'-bipyridine ligands (vinyl linked methoxybenzene or benzo-crown-ether derivatives).³⁷ On the other hand, progressive functionalization of the C_{60} fullerene core decreases its Φ_Δ value, in going from C_{60} to the mono-, bis-, tris-, tetra- and hexa-adducts,^{39,40} despite a constant value of $f_\Delta^{O_2}$ equal to 1, due to a progressive decrease in the Φ_T value.

A study with dicationic, free-base and metallated forms of the hematoporphyrin derivative (HpD), hematoporphyrin IX (Hp9) and a boronated protoporphyrin (BOPP) in methanol, ethanol, acetone and acetonitrile reported values for the free-base form of all the porphyrins and the dicationic forms of Hp9 and HpD in the range 0.44–0.85. Higher Φ_Δ values were obtained for the free-base forms of Hp9 and HpD compared to those of BOPP in the respective solvents. No change in Φ_Δ for the dicationic forms of Hp9 and HpD was observed compared to the values obtained for the free-base forms. However, an almost complete suppression of singlet oxygen production as well as of the fluorescence was noted for the BOPP dication. This unusual observation was attributed to the increased nonplanarity of the macrocycle arising from the steric crowding caused by the four hydrogen atoms in the porphyrin core. Although such steric crowding would also be expected for the dicationic

forms of Hp9 and HpD, the nonplanarity is increased in BOPP by the bulky nature of the peripheral substituents (large *closo*-carborane).⁴¹ Incorporation of Zn(II) ions into the macrocycle reduces Φ_{Δ} for all three porphyrins. BOPP facilitates the coordination of certain transition metals (Mn, Co and Cu) compared to Hp9 and HpD and this results in a dramatic decrease in Φ_{Δ} because of the introduction of low-energy charge-transfer states associated with the disruption of the planarity of the macrocyclic ring, providing alternative nonradiative deactivation pathways.

6.2.6. Electronic Configuration of the Excited State

Rate constants of bimolecular quenching of singlet excited states by O_2 , $k_{s,q}^{O_2}$, may vary over a rather wide range. In general, $k_{s,q}^{O_2}$ are diffusion controlled in condensed phase for most of the sensitizers having an $S_1(\pi\pi^*)$ configuration, while in the case of $S_1(n\pi^*)$ states, a few studies have reported $k_{s,q}^{O_2}$ values smaller than the diffusion-controlled limit.^{42,43}

In the case of triplet excited states, long-lived $T_1(\pi\pi^*)$ states are poorly reactive and exhibit efficient energy-transfer processes to O_2 ; therefore, $P_T^{O_2}$ equal to unity is generally assumed in air-saturated solution. Concerning the efficiency of singlet oxygen production, values of $f_{T,\Delta}^{O_2}$ for $T_1(\pi\pi^*)$ states of aromatic hydrocarbons and ketones are typically in the range 0.8–1, unless E_{T1} is very high or E_{ox} is very low, as previously discussed (Section 6.2.4).^{44,45} On the other hand, in the case of $n\pi^*$ excited monoketones, $f_{T,\Delta}^{O_2}$ values in the range 0.3–0.5 are commonly found, in spite of their often very high E_{ox} .⁴⁶ This behavior has not been found with bi- and triketones or azoalkanes having $n\pi^*$ triplet states, which display $f_{T,\Delta}^{O_2}$ values in the range 0.7–1.^{42,43,47}

6.2.7. Role of Solvent Viscosity and Polarity

Common organic solvents show low viscosities compared to that of water. As previously discussed, $k_q^{O_2}$ decreases with increasing solvent viscosity (η) because of the inverse dependence of the reactants diffusion coefficients on the viscosity of the liquid phase (Section 6.2.3). On the other hand, solvent polarity greatly influences all singlet oxygen production parameters and this has been demonstrated for different classes of sensitizers such as aromatic hydrocarbons, Ru(II) complexes and (tetraphenylporphyrin)zinc(II). In general, an increase in solvent polarity provokes an increase in $k_{T,q}^{O_2}$ and a concomitant decrease in $f_{T,\Delta}^{O_2}$ because of the stabilization of the excited complex $^*(PS\cdots O_2)$ with some charge-transfer character in polar solvents, which opens up new deactivation pathways for PS^* and reduces the efficiency of $^1O_2$ production.^{17,21,24,31,48–57}

The solvent effect may also depend on the ability of the solvent to form hydrogen bonds with the sensitizer (increase in $f_{T,\Delta}^{O_2}$ values).⁵⁸ It has also been noted that the Φ_{Δ} value of aromatic carboxylic acids depends on the pH of the sample since the singlet oxygen yield decreases with deprotonation.^{59,60}

An increase of Φ_{Δ} with pH has been observed in a phthalocyanine, (tetra-*tert*-butylphthalocyaninato)zinc(II), which has been attributed to a decrease of $f_{T,\Delta}^{O_2}$ with increasing protonation that lowers E_T below 94 kJ mol⁻¹.⁶¹

This solvent-dependent behavior is less evident for aromatic ketones with close-lying $\pi\pi^*$ and $n\pi^*$ excited states, whose relative ordering for the singlet and triplet configurations (S_1 vs. T_1 and/or T_2) may vary with solvent polarity, *i.e.*, a given excited state with a certain configuration can be more stable in polar solvents while the situation is reversed in nonpolar ones (*e.g.*, 1*H*-phenalen-1-one (phenalene), 9*H*-fluoren-9-one (fluorenone)). The effect of a series of 18 solvents and mixtures on the production of singlet molecular oxygen by fluorenone has been reported.⁶² Values of Φ_{Δ} for this sensitizer are strongly influenced by the nature of the solvent (1.00 in alkanes, 0.83 in benzene, 0.10 in methanol) and its fluorescence lifetime, quantum yield, and Φ_{ISC} are highly solvent dependent as well. Φ_{Δ} values decrease with increasing solvent polarity and protic character as a consequence of the decrease in Φ_{ISC} . In nonprotic solvents, even of increasing polarities, $P_{T_1}^{O_2}$ and $f_{T,\Delta}^{O_2}$ are close to unity because the triplet lifetime is long enough (0.1 ms in acetonitrile, 1 ms in benzene), and the triplet-quenching mechanism is largely dominated by energy transfer to O_2 . However, when polarity increases, Φ_{ISC} and therefore Φ_{Δ} decrease due to solvent-induced changes in the energy levels of singlet and triplet excited states of the aromatic ketone (in polar solvents $E_{S_1}(\pi\pi^*) < E_{T_2}(n\pi^*)$ and ISC is not favored, while in nonpolar solvents $E_{S_1}(\pi\pi^*) > E_{T_2}(n\pi^*)$ and ISC is favorable). In protic solvents, Φ_{Δ} is low because hydrogen bonding considerably increases the rate of internal conversion from the S_1 state, thus diminishing Φ_{Δ} to values much lower than those in nonprotic solvents of similar polarity. In mixtures of cyclohexane and alcohols, preferential solvation of the sensitizer by the protic solvent leads to a fast decrease of Φ_{Δ} upon addition of increasing amounts of the latter.

Protonation of amine groups of bis(amino)phenylene vinylene sensitizers in water and in toluene has been demonstrated to adversely affect singlet-oxygen production. Although, in a number of cases, protonation-dependent changes in Φ_{Δ} have been attributed to changes in the quantum yield of the sensitizer triplet state (Φ_{ISC}) and to possible changes in the triplet-state energy, other processes play a role in this system: (i) protonation-dependent changes in sensitizer aggregation and (ii) nonradiative channels for sensitizer deactivation that are enhanced as a consequence of the reversible protonation/deprotonation of the chromophore.⁶³

Singlet oxygen production and quenching in microheterogeneous media have been reported for two photostable anionic Ru(II) complexes $[RuL_2L']^{2-}$ (where L stands for (1,10-phenanthroline-4,7-diyl)bis(benzenesulfonate) and L' stands for *N*-(1,10-phenanthroline-5-yl)acetamide or *N*-(1,10-phenanthroline-5-yl)tetradecanamide) and for the cationic complex $[Ru(bpy)_3]^{2+}$.⁶⁴ Comparative results of photosensitized $^1O_2$ generation in micelles, reverse micelles, and microemulsions evidenced that the nature of the ligands and the size and charge of the Ru(II) complexes are important factors affecting their effective location and singlet oxygen production in these media. The

rate constants of quenching of the excited triplet state by O_2 are in the range of $(1-3) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. The emission lifetimes of the excited sensitizers and $k_q^{O_2}$ values are dependent on the nature of the ligand and on the medium. Φ_Δ values in air-equilibrated solutions are between 0.30 and 0.75, and $f_{T,\Delta}^{O_2}$ was demonstrated to be a valuable probe of the interactions of the $Ru(II)$ complexes with micelles and microemulsions since the highest $f_{T,\Delta}^{O_2}$ values (≥ 0.90) were observed in micellar media based on surfactants bearing a charge opposite to that of the $Ru(II)$ complex. In the microheterogeneous systems investigated, the most probable location of the $Ru(II)$ sensitizers is the micellar interfacial region.

6.2.8. Aggregation and Oligomerization of the Excited and Ground States of the Photosensitizer

Sensitizer aggregation, in its excited or ground state, is responsible for lower quantum yields of singlet oxygen production. The role played by excimer formation was demonstrated in a photosensitization study using concentrated solutions of naphthalene and pyrene in methyl alcohol, hexane,⁶⁵ and benzene,⁶⁶ which showed decreased Φ_Δ values compared to their diluted solutions. On the other hand, phenanthrene, which is not able to form excimers, showed a constant Φ_Δ .⁶⁵ More recent studies have shown that $f_{T,\Delta}^{O_2}$ decreases with the degree of charge transfer in the exciplexes,⁶⁷ and that excimer formation in the solid phase is a pathway competitive with $^1O_2$ production.⁶⁸

Formation of ground-state dimers or aggregates has also been reported as the cause of reduced Φ_Δ values for increasing concentrations of bacteriochlorophyll e,⁶⁹ porphyrins,⁷⁰ and hematoporphyrins in water; however, constant values were observed at different sensitizer concentrations in organic solvents such as methanol.⁷¹⁻⁷⁴ This Φ_Δ dependence on the aggregation of sensitizer molecules in certain solvents has also been suggested for tetrakis(4-sulfonatophenyl)porphyrin (TPPS4), (coproporphyrin)zinc(II) and pheophorbides.⁵³ The influence of sulfonation degree and central-atom nature on Φ_Δ has been studied for a series of sulfonated phthalocyanine metal complexes and it was found that in DMSO, where the studied dyes exist as monomers, Φ_Δ values are independent of the number and position of the sulfonate groups. However, in aqueous solutions, aggregation of the dyes determines their photochemical activity.^{75,76} Different types of phthalocyanine oligomers (silicon octaphenoxy-phthalocyanines)⁷⁷ and Ge and Sn phthalocyanines (monomers and μ -oxo-bridged dimers)⁷⁸ have been studied and, in general, a decrease in Φ_Δ has been observed in going from the monomer to the oligomer or dimer (because of lower $f_{T,\Delta}^{O_2}$ values determined for the dimers due to their lower E_T). The ability of Si or Zn phthalocyanines to photosensitize singlet oxygen while decreasing their tendency to aggregate can be significantly improved with dendrimeric structures following the vertical axis or the plane of the chromophore, causing a higher degree of disaggregation in the more branched derivatives.^{79,80}

A study of the effect of NaCl addition to aqueous solutions of tetrakis(4-sulfonatophenyl)porphyrin (TPPS4) forms has shown that the presence of NaCl reduces Φ_{ISC} and τ_T^0 of the biprotonated and nonprotonated TPPS4. The effect originates from Na^+ and/or Cl^- interaction with porphyrin molecules, which directly reduces both triplet lifetime and quantum yield and induces TPPS4 aggregation as well, thus reducing Φ_{ISC} and τ_T^0 even more. The singlet excited-state lifetime and the fluorescence quantum yield also decrease upon NaCl addition. Therefore, interaction of TPPS4 with ions, as well as aggregation, increases the probability of nonradiative dissipation of the TPPS4 electronic excitation. Nevertheless, the quantum yield and the lifetime of TPPS4 J aggregate triplet state are comparable with those of TPPS4 monomers. Production of singlet oxygen is directly proportional to the TPPS4 triplet-state quantum yield and is independent of its lifetime. On the other hand, the difference between Φ_{ISC} and Φ_{Δ} evidences that only a fraction of the TPPS4 triplet states transfer the excitation energy to molecular oxygen producing singlet oxygen. The other fraction is quenched due to different oxygen-dependent mechanisms, probably electron transfer leading to the formation of the superoxide radical. The presence of NaCl affects neither the quenching constant of the TPPS4 triplet state by O_2 nor the lifetime of singlet oxygen.⁸¹

Concerning fullerenes, formation of the C_{60} fullerene triplet state depends on its aggregation state since it has been demonstrated that the Φ_T value depends on the C_{60} concentration, reducing gradually with an increase in its concentration.⁸² Nano- and microaggregates of C_{60} fullerene have shown their ability to photosensitize $^1O_2$ generation in their interior. However, despite the efficient photosensitization and long lifetimes of singlet oxygen that can be achieved (due to $^1O_2$ insensitivity to solvent and quenchers) potential applications based, for instance, in $^1O_2$ photocytotoxicity, could not be efficiently developed.^{83,84}

6.2.9. Effects of Temperature and Pressure

A study on the dependence of $k_{T,q}^{O_2}$ values of several ketones on the temperature in condensed phase (toluene solution) showed a linear increase of $\ln(k_{T,q}^{O_2})$ with $1/T$ in the 300–360 K interval, while at 300 K a turnover point was found and at lower temperatures $\ln(k_{T,q}^{O_2})$ decreases with $1/T$.^{85,86} The observed behavior was attributed to a change from pre-equilibrium to diffusion control in the triplet-state quenching by O_2 . A T -dependent study of the quenching of the naphthalene triplet by O_2 in several solvents showed that the activation energy (E_a) changed from slightly positive values in n -hexane and methylcyclohexane to negative ones in acetone, acetonitrile and toluene. The negative E_a values were justified by the formation of exciplexes.⁸⁷ Dependence of $f_{T,\Delta}^{O_2}$ on T was also demonstrated in toluene, and a different behavior was observed for chrysene where $f_{T,\Delta}^{O_2}$ increased with T and for benzoylbiphenyl and phenalenone where $f_{T,\Delta}^{O_2}$ decreased with increasing T .⁸⁸

Concerning the influence of pressure on singlet oxygen production, some studies using several aromatic sensitizers in different solvents have demonstrated that $k_{s,q}^{O_2}$ and $k_{t,q}^{O_2}$ decrease with increasing pressure.^{89–93} The dependence of $k_{s,q}^{O_2}$ on pressure is more important than that of $k_{t,q}^{O_2}$ since $k_{s,q}^{O_2}$ is assumed to be nearly diffusion controlled. In this sense, $\ln(k_{s,q}^{O_2})$ correlates linearly and inversely with pressure and with $\ln(\eta)$, as a result of increasing solvent viscosity with increasing pressure. On the other hand, correlations of $\ln(k_{t,q}^{O_2})$ with $\ln(\eta)$ are nonlinear and with a smaller slope, in agreement with the nondiffusional nature of the $^3PS^*$ quenching by O_2 at room temperature and atmospheric pressure.

6.3. General Features of a Reference Singlet Oxygen Photosensitizer

Most commonly used standards for singlet oxygen production are commercially available or easy to prepare organic or metal-organic photostable molecules, with intense absorption bands in the UV-vis region, which are able to photosensitize singlet oxygen with high Φ_Δ values in a wide variety of organic solvents and, eventually, also in water. An ideal sensitizer should be, therefore, thermally and photochemically stable, and have a solvent-independent and high Φ_Δ . Moreover, standard photosensitizers should have a low rate constant of singlet oxygen quenching by the sensitizer itself ($k_{\Delta,q}^{PS}$), benefiting from the absence of self-sensitization of its own photo-oxidation and enabling the use of rather high sensitizer concentrations.

Singlet oxygen photogeneration by ST and TC sensitizers is strongly influenced by: (i) the balance between the competitive deactivation pathways of the S_1 state (*i.e.*, fluorescence, internal conversion, quenching by $^3O_2$ leading to $^1O_2$, when $(E_{S1} - E_{T1}) > E_\Delta$ or, when $(E_{S1} - E_{T1}) < E_\Delta$, enhanced ISC leading to excited T_2 or T_1 states) and (ii) by solvent effects (*e.g.*, reordering of $\pi\pi^*$ and $n\pi^*$ energy levels). Therefore, $^1O_2$ photosensitization using ST and TC sensitizers is likely to be much more complex and more dependent on the O_2 concentration than $^1O_2$ photosensitization by T-type sensitizers. Thus, singlet oxygen production standards are, generally, triplet sensitizers showing a Φ_T value close to unity in as many solvents as possible.

Due to the spin-forbidden character of the intramolecular $T_1 \rightarrow S_0$ transition, T_1 states of reference sensitizers usually display long lifetimes (τ_T^0 in the 10 μs –100 ms range), that allow almost complete quenching by ground-state molecular oxygen even in air-equilibrated solutions and despite the fact that $k_{t,q}^{O_2}$ is typically one order of magnitude lower than the diffusion-controlled limit. Therefore, $P_T^{O_2}$ values of standard sensitizers for singlet oxygen production are, in general, close to unity.

The most delicate parameter is the efficiency of singlet oxygen production ($f_{T,\Delta}^{O_2}$) that is strongly dependent on the solvent nature (nonpolar and protic or aprotic polar solvents) since the influence of charge-transfer interactions in the excited encounter complex controls the balance between the competitive

deactivation pathways resulting or not in efficient singlet oxygen production, as previously discussed. In general, the $f_{T,\Delta}^{O_2}$ parameter is higher in nonpolar solvents for many sensitizers (while $k_{T,q}^{O_2}$ tends to decrease); however, this dependence is less evident in aromatic ketones, making them good candidates as singlet oxygen reference sensitizers with usually high Φ_Δ values.

6.4. Phenalenone, the Universal Reference Compound for the Determination of Quantum Yields of Singlet Oxygen Production

1*H*-Phenalen-1-one, also called 1*H*-benzonaphthen-1-one, 7-perinaphthene or phenalenone, is an aromatic ketone soluble in polar protic and aprotic solvents, as well as in nonpolar media. Its photophysical and singlet oxygen photosensitization properties were thoroughly studied by Oliveros and Braun in 1991. They found that phenalenone was fairly photostable and one of the most efficient singlet oxygen sensitizers in both polar and nonpolar media, and therefore proposed this molecule as a reference sensitizer, in particular in the area of relatively high energies of excitation.⁷ The lowest singlet and triplet excited states of phenalenone have dominant $\pi\pi^*$ electronic configurations and this aromatic ketone is photostable in benzene, hexane and methylcyclohexane, while it can react by H-abstraction in deoxygenated alcohols (*e.g.*, propyl and ethyl alcohols) with low quantum yields of photoreduction ($\Phi_{-H} \leq 0.05$).⁹⁴ Therefore, phenalenone photoreactions do not compete, in general, with the deactivation of its $\pi\pi^*$ triplet state by energy transfer in the presence of O_2 . The ground-state absorption spectrum of phenalenone in polar and nonpolar solvents extends up to about 460 nm and shows two main peaks above 300 nm, which remain almost unchanged upon increasing the polarity of the solvent: 354 nm ($\epsilon = 10\,150\text{ M}^{-1}\text{ cm}^{-1}$) and 377 nm ($\epsilon = 7870\text{ M}^{-1}\text{ cm}^{-1}$) in hexane, 360 nm ($\epsilon = 11\,250\text{ M}^{-1}\text{ cm}^{-1}$) and 382 nm ($\epsilon = 9630\text{ M}^{-1}\text{ cm}^{-1}$) in methanol. The lifetime of the singlet excited state of phenalenone was assumed to be shorter than 5 ps since its fluorescence could not be observed, while the phosphorescence lifetime was found to be 11 ms at 77 K in methylcyclohexane. The lifetimes of the triplet excited state (τ_T^0) at room temperature are 38 μs in benzene and 34 μs in methanol, respectively, with rate constants of quenching by O_2 ($k_{T,q}^{O_2}$) in the range $(1.3\text{--}3.2) \times 10^9\text{ M}^{-1}\text{ s}^{-1}$, close to 1/9 of the diffusional value.⁴⁵ The lowest singlet excited state of phenalenone was reported to have a dominant $\pi\pi^*$ character, with an energy of approx. 265 kJ mol⁻¹ in acetonitrile,^{44,45} while that of the $T_1(\pi\pi^*)$ state is 182 kJ mol⁻¹ in the same solvent. Therefore, $(E_{S1} - E_{T1}) \leq E_\Delta$ and, since the extremely short singlet excited-state lifetime does not allow for efficient quenching by O_2 , phenalenone can be classified as a T-type photosensitizer exhibiting a $T_1(\pi\pi^*)$ state in polar and apolar media.

The remarkably high quantum yield of intersystem crossing (Φ_{ISC}) of phenalenone, equal to unity (with $k_{ISC} \geq 10^8\text{ s}^{-1}$), was explained by the strong spin-orbit coupling between two states of different electronic configurations,

$S_1(\pi\pi^*)$ and a $T_2(n\pi^*)$, lying below $S_1(\pi\pi^*)$. Indeed the large energy gap existing between the $S_1(\pi\pi^*)$ and $T_1(\pi\pi^*)$ states (*ca.* 80–100 kJ mol⁻¹) and the rigidity of the molecule would prevent an efficient $S_1(\pi\pi^*) \rightarrow T_1(\pi\pi^*)$ transition. This fact was confirmed by Flors and Nonell, who reported an energy of 288 kJ mol⁻¹ for the $S_1(\pi\pi^*)$ state and of 185 kJ mol⁻¹ for the $T_1(\pi\pi^*)$ state in methylcyclohexane, and an energy gap of 9 kJ mol⁻¹ between the $T_2(n\pi^*)$ and $T_1(\pi\pi^*)$ excited states.^{95,96} This large energy gap between S_1 and T_2 makes it unlikely that the two states reverse their energy by increasing solvent polarity (that could change the balance between the competitive deactivation pathways of excited phenalenone). Therefore, a Φ_{ISC} very close to 1 should be expected in all solvents.

Recent results based on theoretical calculations, in good agreement with previous experimental results, have allowed a better knowledge of the nature of the lowest excited states of phenalenone, its intersystem crossing process, and its singlet oxygen production ability as well.^{97,98} In a first study, without inclusion of solvent effects, the lowest excited singlet states were estimated to be of $S_1(n\pi^*)$ and $S_2(\pi\pi^*)$ type, with energies of *ca.* 212 kJ mol⁻¹ and *ca.* 287 kJ mol⁻¹, respectively (considering the zero-point vibrational energy). Energetically accessible from $S_1(n\pi^*)$ are two triplet states with $T_1(\pi\pi^*)$ and $T_2(n\pi^*)$ configurations, and energies of *ca.* 168 kJ mol⁻¹ and *ca.* 209 kJ mol⁻¹, respectively, the latter being nearly degenerated with $S_1(n\pi^*)$.⁹⁷ Intersystem crossing between $S_1(n\pi^*)$ and $T_1(\pi\pi^*)$ was estimated to be very efficient, with a calculated ISC rate constant of *ca.* 2×10^{10} s⁻¹, in very good agreement with the experimental value of 3.45×10^{10} s⁻¹.⁹⁹ A second study using *ab initio* quantum chemical calculations allowed the elucidation of the mechanism for populating the triplet $T_1(\pi\pi^*)$ state responsible for the reactivity of excited phenalenone (*i.e.*, energy transfer to molecular oxygen or hydrogen abstraction from protic solvents).⁹⁸ Computational results showed that, after the initial population of the short-lived $S_2(\pi\pi^*)$ excited state, whose radiative deactivation is allowed but with low probability of luminescence (in agreement with the experimental quantum yield of fluorescence of 10^{-4}), the system undergoes an almost barrierless internal conversion to the $S_1(n\pi^*)$ dark state, which relaxes to its minimum but rapidly populates the triplet manifold after a very efficient ISC to the $T_1(\pi\pi^*)$ state through an avoided crossing only 0.31 eV (30 kJ mol⁻¹) above the $S_1(n\pi^*)$ minimum. Although the population of the minimum of this triplet state is strongly favored (phosphorescence from this minimum is predicted to show a wavelength of 1.72 eV, in reasonable agreement with the experimental value of 1.91 eV), a conical intersection with the $T_2(n\pi^*)$ surface opens up an internal conversion channel at high temperatures to the reactive $T_2(n\pi^*)$ state responsible for the hydrogen-abstraction ability of phenalenone. The equilibrium between both triplet species will depend on the temperature, given that the $T_1(\pi\pi^*)$ minimum is sufficiently long lived to reach the thermal equilibrium with the environment, losing the excess of excitation energy. Radiationless deactivation processes can be ruled out on the basis of the high-energy barriers found for the crossings between the excited states and the ground-state surfaces.

The computational results confirmed that the $T_1(\pi\pi^*)$ state is not only the most stable excited species but that it is also characterized by a deep well surrounded by relatively high barriers (0.81 eV to the intersystem crossing, 0.68 eV to the internal conversion), which makes the $T_1(\pi\pi^*)$ long lived and explains the fact that phenalenone has a large quantum yield for the sensitization of singlet oxygen formed from the $T_1(\pi\pi^*)$ state.

The energy-transfer efficiency from the phenalenone triplet state to molecular oxygen ($f_{T,\Delta}^{O_2}$) is also close to unity. Therefore, according to the values of Φ_{ISC} , $P_T^{O_2}$ and $f_{T,\Delta}^{O_2}$ of phenalenone, this sensitizer must definitely be among the most efficient singlet oxygen sensitizers of $\pi\pi^*$ electronic configuration. The quantum yield of singlet oxygen production by phenalenone is close to 1, both in polar solvents ($\Phi_\Delta = 0.97 \pm 0.03$ in deuterated methanol using rose bengal as the reference, $\Phi_\Delta = 0.76$) and nonpolar solvents ($\Phi_\Delta = 0.93 \pm 0.04$ in benzene and deuterated benzene using 9H-fluoren-9-one as the reference, $\Phi_\Delta = 0.825$). Furthermore, a quite remarkable feature of phenalenone is its negligible quenching of the photogenerated singlet oxygen by its own ground state ($k_{\Delta,q}^{PS} = 3.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for phenalenone in perfluorodecalin,⁴⁴ similar to $k_{\Delta,q}^{PS} = 6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for rose bengal in methanol), which allows for a Φ_Δ value independent of the photosensitizer concentration.

In 1994 Schmidt and Tanielian,⁴⁴ proposed phenalenone as the universal reference compound for the determination of quantum yields of singlet-oxygen sensitization in solution by comparative techniques. Values of Φ_Δ were determined in 13 solvents covering almost the entire polarity scale and very different physical and chemical properties (water, acetonitrile, methanol, acetone, 2-propanol, tetrahydrofuran, bromobenzene, chloroform, iodobenzene, toluene, benzene, tetrachloromethane and cyclohexane) and in methanol–water systems as well. The $T_1(\pi\pi^*)$ state of phenalenone is completely quenched by O_2 in air-equilibrated organic solutions of moderate viscosity and, since the solubility of O_2 in water is one order of magnitude smaller than in organic solvents, the authors recommended oxygen saturation in the case of aqueous solutions of phenalenone, in order to avoid reduced efficiencies of singlet oxygen generation due to incomplete T_1 state quenching. Values of Φ_Δ were determined by photochemical, photoacoustic and near-IR luminescence methods and it was observed that Φ_Δ was wavelength-independent in the 337–436 nm range, and varied only in a narrow range $0.94 < \Phi_\Delta < 1.00$ for the different solvents tested. Φ_Δ was independent of the phenalenone concentration up to $2.7 \times 10^{-3} \text{ M}$. Therefore, it was proposed that extrapolations to air-saturated organic solvents and to O_2 -saturated micellar systems of low viscosity should be possible assuming $\Phi_\Delta = 0.95 \pm 0.05$. Quantum yields of phenalenone bleaching in 2-propanol and methanol were determined to be $\leq 8 \times 10^{-3}$ and $\leq 5 \times 10^{-3}$, respectively. However, higher quantum yields of H-abstraction were reported in dioxane (0.013) and *N,N'*-dimethylacetamide (0.023), while singlet oxygen photo-generation remained efficient ($\Phi_\Delta = 0.99 \pm 0.05$ in dioxane and $\Phi_\Delta = 0.87 \pm 0.05$ in *N,N'*-dimethylacetamide).¹⁰⁰

Table 6.1. Quantum yields of singlet oxygen production by phenalenone (1) in different air-equilibrated solvents.

Cyclohexane 0.91 ± 0.03 ⁴⁵	Benzene 0.93 ± 0.04 ⁷	Toluene 0.92 ± 0.03 ⁴⁵	Dichloromethane 0.96 ± 0.08 ⁴⁵	1,4-Dioxane 0.99 ± 0.05 ¹⁰⁰
<i>N,N'</i> -Dimethylac- etamide 0.87 ± 0.05 ¹⁰⁰	Acetonitrile 1.00 ± 0.03 ⁴⁵	Ethanol 0.92 ± 0.03 ⁴⁵	Methanol 0.97 ± 0.04 ⁷	Water 0.98 ± 0.08 ⁴⁴

The potential of phenalenone as a standard for singlet oxygen photosensitization was further confirmed by Nonell *et al.* in 1996.⁴⁵ The authors compared the Φ_{Δ} values of several aromatic ketones: phenalenone and its water-soluble 2-sulfonic acid derivative,¹⁰¹ benzanthrone, 4-phenylbenzophenone and the benzophenone–naphthalene (0.1 M) system. In order to assess their adoption as solvent-independent standards for singlet oxygen photosensitization, time-resolved near-IR emission and optoacoustic calorimetry studies were carried out and all compounds showed Φ_{Δ} values in the range 0.9–1 in cyclohexane. However, increasing solvent polarity or protic character reduced the Φ_{Δ} values for all sensitizers except phenalenone for which highly precise absolute Φ_{Δ} values in the range 0.91–1 were determined, with recommended values of Φ_{Δ} showing uncertainties generally not exceeding 3%. Therefore, phenalenone can be considered as a reliable standard that researchers can always trust when evaluating the quantum yield of photosensitized singlet oxygen production by any new photosensitizer. Table 6.1 collects the quantum yields of singlet oxygen production by phenalenone in different solvents.

6.5. Other Reference Singlet Oxygen Photosensitizers

Other well-known singlet oxygen sensitizers have often been used as standards, employing either direct (mainly luminescence at 1270 nm) or indirect (chemical probes) singlet oxygen detection methods. It has to be noted, however, that special care has to be taken when methods based on chemical probes are used for the determination of unknown Φ_{Δ} values, unless it is proven that a complete electron-transfer pathway (yielding radical ions) does not compete with energy transfer to O₂, neither for the reference nor for the sensitizer under study. As previously mentioned, the choice of the standard for determining Φ_{Δ} of a new sensitizer or of a known sensitizer under different conditions relies on the following main parameters, in order to minimize the number of corrections in the calculations: high Φ_{Δ} value, photostability, adequate and (quasi)monochromatic excitation wavelength, the same absorbance at the wavelength(s) of excitation for probe and reference, similar incident photon fluxes if the two samples are excited at different wavelengths, and solubility in the same solvent for the standard and the sensitizer under evaluation. Moreover, in comparative experiments, the sensitizer under evaluation and the reference must be investigated using the same experimental

conditions and the linearity of the response as a function of the excitation energy has to be verified for both samples. Since most of these methods are based on the comparison between the $^1\text{O}_2$ phosphorescence signal obtained with the standard and the sensitizer under evaluation, accurate knowledge of the Φ_{Δ} of the standard is required. In the following, established reference systems for which Φ_{Δ} is known with a relative precision of about $\pm 10\%$ in a limited selection of solvents (*e.g.*, benzene/toluene, (cyclo)alkanes, dichloromethane, 1,4-dioxane, acetone, acetonitrile, ethyl/methyl alcohol and water) are gathered according to their family, and their possible drawbacks are mentioned. For other solvents, the relative uncertainty of the Φ_{Δ} values is much larger ($\pm 20\% - \pm 25\%$).⁴⁴

6.5.1. Polynuclear Aromatic Hydrocarbons

It has long been known that polyaromatics, such as substituted anthracene,^{16,17} naphthalene,²⁵ and biphenyl derivatives,²¹ were able to efficiently sensitize singlet oxygen production, from both their singlet and triplet excited states,¹⁰² and the series of cyanoaromatics (such as 9,10-dicyanoanthracene, a typical ST sensitizer) deserved special attention.^{17,103-105} However, due to the efficient quenching of both singlet and triplet states by ground-state molecular oxygen, singlet oxygen quantum yields are generally very sensitive to the oxygen concentration. Moreover, the competition between energy transfer and charge transfer, depending both on the intrinsic features of the sensitizer (singlet and triplet energy, oxidation potential, *etc.*) and on the solvent makes them rather difficult to use as reference sensitizers. Finally, some of these polynuclear aromatic hydrocarbons (alkyl or aryl-9,10-substituted anthracenes for instance) tend to easily form photoadducts with singlet oxygen, resulting in fast bleaching of the samples, a feature that makes these aromatic hydrocarbons alternatively useful as singlet oxygen scavengers.¹⁰⁶

6.5.2. Aromatic Ketones and Quinones

Aromatic ketones are good triplet sensitizers due to their easily formed $n\pi^*$ or $\pi\pi^*$ triplet states and, among them, biologically active quinones and hydroxyquinones have been shown to produce singlet oxygen efficiently in aprotic solvents (Φ_{Δ} 0.38 and 0.62–0.70 in acetonitrile for benzoquinone and anthraquinone, respectively, for an excitation at 337 nm).¹⁰⁷ However, they are also moderate $^1\text{O}_2$ quenchers (rate of physical quenching $10^6 - 10^7 \text{ M}^{-1} \text{ s}^{-1}$). Since triplet lifetimes and quantum yields depend on the $n\pi^*$ or $\pi\pi^*$ nature of the excited states, the singlet oxygen quantum yields of quinones is greatly affected by the position of substituents (in position 1 or 2) and by the use of protic solvents, which can modify the relative levels of these $n\pi^*$ or $\pi\pi^*$ states.

Other aromatic ketones than phenalenone have been evaluated as potential standards under excitation wavelengths in the UV range and it was shown

that the solvent dependence of the Φ_{Δ} values for benzantrone, 4-phenylbenzophenone and the system (benzophenone + naphthalene) ruled them out as universal standards compared to phenalenone, even if high singlet-oxygen quantum yields were determined in a range of solvents.⁴⁵ An example of aromatic ketone with a $\pi\pi^*$ triplet state in polar and apolar solvents is 2-acetonaphthone for which high Φ_{Δ} values were measured both in benzene ($\Phi_{\Delta} 0.73 \pm 0.4$) and in methanol ($\Phi_{\Delta} 0.79 \pm 0.4$) for $\lambda_{\text{exc}} 337 \text{ nm}$.⁷ On the other hand, fluorenone (Section 6.2.7) is a good reference sensitizer that can be used in nonpolar aprotic solvents such as alkanes and cycloalkanes where Φ_{Δ} is equal to 1.0 ± 0.05 , and fluorenone is stable under irradiation in these solvents in the presence of O_2 .⁶² Two new derivatives of phenalenone were also proposed as suitable singlet oxygen standards for both 1- and 2-photon absorption (see Section 6.5.7).¹⁰⁸ Table 6.2 collects the quantum yields of singlet oxygen production and the photophysical properties of some aromatic ketones that have been used as standards for singlet oxygen photogeneration in different solvents.

6.5.3. Heterocycles: Rose Bengal, Methylene Blue and Acridine

The photophysical and photochemical properties of heavy atom-substituted xanthene dyes such as rose bengal are notable for their strong absorption in the visible range (490–560 nm), high triplet quantum yields (≈ 1) and for triplet states with fairly long lifetimes from 0.1 to 0.3 ms.¹⁰⁹ The rose bengal sodium salt (RB), is a popular reference sensitizer soluble in polar solvents. Its most often cited value of Φ_{Δ} (0.76 ± 0.02 in MeOH or neutral water for λ_{exc} between 530 and 555 nm) is higher than for any other fluorescein derivative dye.^{1,110} However, RB is susceptible to (photo)bleaching in polar protic solvents and also slightly acidic solutions, which has to be controlled for long-lasting experiments, and it can aggregate in water between 10^{-6} and 10^{-3} M .¹¹¹

The phenothiazinium dyes exhibit intense absorption maxima in the 600–660 nm region of the spectrum (typically $\epsilon > 50\,000 \text{ M}^{-1} \text{ cm}^{-1}$) useful in photodynamic therapy. Among them the well-known cationic dye, methylene blue, is also often cited as a standard for singlet oxygen production in polar solvents (Φ_{Δ} in the range 0.5–0.6 in MeOH, EtOH or neutral water, for λ_{exc} between 630 and 660 nm).^{1,112} However, its aggregation state has to be carefully controlled.¹¹³

Acridine has been recommended as a standard for comparative measurements of singlet oxygen production due to the efficient intersystem crossing from the singlet excited state to the triplet state ($\Phi_{\text{ISC}} = 0.84$ in benzene), a $f_{\text{T},\Delta}^{\text{O}_2}$ close to unity and its stability upon photolysis in hydrocarbon solvents and under free-radical polymerization conditions. High singlet oxygen quantum yields were reported in nonpolar solvents for acridine ($\Phi_{\Delta} = 0.73 \pm 0.13$ in toluene, and $\Phi_{\Delta} = 0.83 \pm 0.06$ in benzene for $\lambda_{\text{exc}} 355 \text{ nm}$).^{1,114} Table 6.3 collects the quantum yields of singlet oxygen production and the photophysical

Table 6.2. Quantum yields of singlet oxygen production in air-equilibrated solutions and photophysical properties of aromatic ketones used as standards.^{a,b,c}

Sensitizer	Solvent	$\Phi_{\Delta} \pm \text{Std dev}$	f_{Δ}^T	$\lambda_{\text{exc}}/\text{nm}$	$E_s/\text{kJ mol}^{-1}$	$E_T/\text{kJ mol}^{-1}$	Φ_T	$\tau_T^0/\mu\text{s}$	$k_q^{\text{O}_2}/\text{M}^{-1} \text{s}^{-1}$
2	Benzene	0.73 ± 0.04^7	0.87^7	337	325^9	249^9	0.84^9	300^9	$1.7\text{--}2.5 \times 10^9 \text{ n, p}^9$
	Methanol	0.79 ± 0.04^7							
3	Cyclohexane	0.84 ± 0.06^{45}	—	337	342 n^9	255 n^9	1 n^9	40 n^{161}	—
	Toluene	0.95 ± 0.07^{45}							
	Dichloromethane	0.78 ± 0.06^{45}							
	Acetonitrile	0.78 ± 0.06^{45}							
	Ethanol	0.75 ± 0.06^{45}							
4	Cyclohexane	0.94 ± 0.07^{45}	—	337	—	192 p^9	—	—	—
	Toluene	0.88 ± 0.06^{45}							
	Dichloromethane	0.80 ± 0.06^{45}							
	Acetonitrile	0.83 ± 0.06^{45}							
	Ethanol	0.76 ± 0.06^{45}							
5	Cyclohexane	1.00 ± 0.05^{62}	$\approx 1^{62}$	337, 367	266 p^9	211 p^9	1.03^{62}	1000 n^{62}	—
	Heptane	1.00 ± 0.05^{62}					0.94 n^9		
	Benzene	0.83 ± 0.04^{62}					0.93^{62}		
	1,4-Dioxane	0.92 ± 0.05^{62}					0.96^{62}		
	Acetone	0.79 ± 0.04^{62}					0.77^{62}		

^aEntries: 2 = 2-acetonaphthone, 3 = 4-phenylbenzophenone, 4 = benzanthrone, 5 = 9H-fluoren-9-one.^bn = nonpolar, benzene-like solvent.^cp = polar, ethanol-like solvent.

Table 6.3. Quantum yields of singlet oxygen production in air-equilibrated solutions and photophysical properties of heterocycles and C₆₀ fullerene used as standards.^{a,b,c}

Sensitizer	Solvent	$\Phi_{\Delta} \pm \text{Std dev}$	f_{Δ}^T	$\lambda_{\text{exc}}/\text{nm}$	$E_S/\text{kJ mol}^{-1}$	$E_T/\text{kJ mol}^{-1}$	Φ_T	$\tau_T^0/\mu\text{s}$	$k_q^{O_2}/\text{M}^{-1} \text{s}^{-1}$
6	Benzene	0.83 ± 0.06^1	0.99 ± 0.02^1	355	315 p ⁹	190 ⁹	0.82 p ⁹	10 000 n ⁹	$1.8 \times 10^9 \text{ n}^9$
7	Methanol	0.51 ± 0.01^1	1.00 ± 0.01^1	630–660	180 p ⁹	138 p ⁹	0.52 p ⁹	450 p ⁹	$1.7 \times 10^9 \text{ p}^9$
	Water	0.55 ± 0.03^1							
8	Methanol	0.76 ± 0.02^{110}	1.00 ± 0.01^1	530–555	213 p ⁹	164 p ⁹	0.90 ± 0.08^{110}	130 ¹¹⁰	$9.8 \times 10^8 \text{ p}^9$
	Water	0.75 ± 0.02^{110}					1.05 ± 0.06^{110}	160 ¹¹⁰	
9	Benzene	1.01 ± 0.03^{117}	1.0 ± 0.1^1	510	193 n ⁹	151 n ⁹	1 n ⁹	250 n ⁹	$1.9 \times 10^9 \text{ n}^9$
		0.98 ± 0.05^{118}							

^aEntries: 6 = acridine, 7 = methylene blue cation, 8 = rose bengal dianion; 9 = C₆₀ fullerene.

^bn = nonpolar, benzene-like solvent.

^cp = polar solvent.

properties of some heterocyclic compounds that have been used as standards for singlet oxygen photogeneration in different solvents.

6.5.4. Carbon Nanoforms: C_{60} Fullerene

Fullerenes are very attractive molecules absorbing photons throughout the entire UV and visible spectrum (200–700 nm). They undergo intersystem crossing to a long-lived triplet state with *ca.* unit efficiency because of the low energy gap between the lowest excited singlet and triplet states (~ 23 kJ mol⁻¹),¹¹² and also are good electron acceptors, highly stable towards oxidation but poorly soluble in polar solvents.¹¹⁵ Singlet oxygen quantum yields first obtained for C_{60} fullerene ($\Phi_{\Delta} = 0.96$ for λ_{exc} 532 nm and 0.76 for λ_{exc} 355 nm in benzene),¹¹⁶ were later shown to be almost independent from the excitation wavelength ($\Phi_{\Delta} = 0.92 \pm 0.05$, 0.98 ± 0.05 and 1.01 ± 0.03 for λ_{exc} 530, 340 and 510 nm, respectively).^{117,118} The quantum yields of singlet oxygen production and the photophysical properties of C_{60} fullerene in benzene are given in Table 6.3. Other more soluble fullerene dihydroderivatives of the *o*-quinodimethane type display lower Φ_{Δ} values (average $\Phi_{\Delta} = 0.76$ in CH_2Cl_2 for λ_{exc} 532 nm) relative to the parent C_{60} while the water-soluble inclusion complexes with γ -cyclodextrin retained much of the photosensitization ability ($\Phi_{\Delta} = 0.77$ in D_2O for λ_{exc} 532 nm) of the parent C_{60} fullerene.¹¹⁵

6.5.5. Porphyrins, Phthalocyanines and Their Metal Complexes, BODIPYs

Porphyrins and their numerous tunable derivatives, soluble either in non-polar or polar solvents, show four absorption bands in the visible range and one very intense Soret band in the near-UV (400 nm). Formation of a metalloporphyrin complex results, in most cases, in the decrease of the number of absorption bands in the visible range from four to two, while the Soret band remains usually unaltered. In apolar solvents, one of the most well-known soluble compounds is 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine (TPP), for which a value of Φ_{Δ} of 0.78 ± 0.04 for λ_{exc} 355 nm in benzene has been reported,¹¹⁹ correcting a previous lower average value (Φ_{Δ} 0.66 ± 0.08 for λ_{exc} 347 or 590 nm in benzene).¹ Metallocomplexes of porphyrins with diamagnetic metal ions such as In(III), Al(III), Zn(II) that extend the triplet lifetimes are also efficient singlet oxygen sensitizers, and one the most used is (tetraphenylporphyrin)zinc(II) with a Φ_{Δ} value of 0.72 ± 0.08 for λ_{exc} 347 or 590 nm in benzene.^{1,120} A large amount of data on water-soluble porphyrinoid sensitizers noncovalently bound to biologically relevant host molecules have been gathered, with reference to common ionic porphyrin derivatives,¹²¹ such as the tetrakis(4-sulfonatophenyl)porphyrin, TPPS4, often used as a standard with a Φ_{Δ} value of 0.62 ± 0.03 and its Zn(II) complex, ZnTPPS4 ($\Phi_{\Delta} = 0.74 \pm 0.07$ for λ_{exc} 532–543, or 347 nm at pH 7).^{1,122} Hematoporphyrin (2,18-dipropionic acid, 7,12-bis(1-hydroxyethyl)-3,8,13,17-tetramethylporphine) and the hematoporphyrin derivative (HpD) were extensively used as first-generation

sensitizers in photodynamic therapy and, therefore, were characterized under a variety of experimental conditions, but with a high degree of scatter in Φ_{Δ} values,¹ probably related to the possible formation of aggregates.^{123,124} However, photobleaching is still one of the main drawbacks of some of these compounds. Table 6.4 collects the quantum yields of singlet oxygen production and the photophysical properties of some porphyrin derivatives and their Zn(II) complexes that have been used as standards for singlet oxygen photogeneration in different solvents.

In the phthalocyanine series presenting intense absorption bands around 700 nm (Q band) and 350 nm (Soret band), the most efficient singlet-oxygen production is obtained from metallophthalocyanines with diamagnetic Al(III) or Zn(II) metal ions. However, due to lower singlet oxygen quantum yields than those of porphyrins, their easy aggregation and overall rather scarce data, they cannot be considered as reference sensitizers.^{1,125,126}

Since the end of the 1980s, a new class of boron complexes (BODIPYs), structural analogs of porphyrins, are holding great promise as ideal sensitizers, specially for photodynamic therapy applications when incorporating halogen atoms (I or Br).^{127–129} High singlet oxygen quantum yields were obtained for lab-made iodinated derivatives ($\Phi_{\Delta} = 0.83–0.87$ in acetonitrile for λ_{exc} 532 nm for three different iodinated compounds).¹²⁸ However, up to now, such BODIPY molecules are not commercially available and no comparative reliable quantum yields have been obtained for these compounds, even if scarce studies showed rather efficient singlet oxygen generation without always accurate determination of the quantum yields.¹³⁰

6.5.6. Coordination Compounds of Transition Metals with Polyazaheterocyclic Ligands

Coordination compounds of Ru(II) with polyazaheteroaromatic ligands have a unique combination of their spectroscopic, redox and photochemical features, together with the possibility of fine tuning their properties by judicious choice of the chelating ligands. Their excitation in the 180–550 nm range results in a fast intersystem crossing leading to the formation of a metal-to-ligand charge-transfer triplet (³MLCT) excited state that may be quenched by ground-state oxygen among other possibilities.³¹ Tris(2,2'-bipyridyl)ruthenium(II) dichloride ([Ru(bpy)₃]Cl₂) belongs to this family of stable sensitizers able to react both by energy transfer and by electron transfer and, as previously stated, this has to be taken into account when the detection method employed for ¹O₂ quantification makes use of chemical probes (Section 6.5). [Ru(bpy)₃]²⁺ is an efficient singlet oxygen sensitizer in polar solvents such as methanol and DMF ($\Phi_{\Delta} = 0.73 \pm 0.06$ for λ_{exc} 367, 437 or 480 nm in methanol).^{31,131} Other commercially available Ru(II) complexes that are even more efficient singlet oxygen photosensitizers than [Ru(bpy)₃]²⁺, are [Ru(dip)₃]²⁺ (dip: 4,7-diphenyl-1,10-phenanthroline) and [Ru(dpds)₃]⁴⁺ (dpds: 4,7-diphenylsulfonate-1,10-phenanthroline), with Φ_{Δ} values of 0.97 ± 0.08

Table 6.4. Quantum yields of singlet oxygen production in air-equilibrated solutions and photophysical properties of some porphyrin derivatives and their Zn(II) complexes used as standards.^{a,b,c}

Sensitizer	Solvent	$\Phi_A \pm \text{Std dev}$	f_A^T	$\lambda_{\text{exc}}/\text{nm}$	$E_S/\text{kJ mol}^{-1}$	$E_T/\text{kJ mol}^{-1}$	Φ_T	$\tau_T^0/\mu\text{s}$	$k_q^0/\text{M}^{-1} \text{s}^{-1}$
10	Benzene	0.78 ± 0.04 ¹¹⁹	1.0 ± 0.1 ¹¹⁹	355	179 n ⁹	138 n ⁹	0.82 n ⁹	1500 n ⁹	2.1×10^9 n ⁹
11	Water	0.62 ± 0.03 ¹²²	0.82 ± 0.01 ¹²²	347 or 532–543	—	—	0.76 p ¹²²	414 p ¹²²	2.0×10^9 p ¹²²
12	Water	0.90 ± 0.02 ¹⁶²	1.0 ± 0.1 ¹⁶²	347	177 p ⁹	—	0.92 p ⁹	170 ¹⁶³	1.9×10^9 p ¹⁶²
13	Benzene	0.72 ± 0.08 ¹	0.7 ± 0.1 ¹	347 or 590	198 n ⁹	153 n ⁹	0.88 n ⁹	1200 n ⁹	1.5×10^9 p ⁹
14	Water	0.74 ± 0.07 ¹²²	0.86 ± 0.1 ¹²²	347 or 532–543	—	—	0.86 p ¹²²	2040 p ¹²²	1.3×10^9 p ¹²²
15	Water	0.88 ± 0.1 ¹	—	546	191 p ⁹	—	0.82 p ⁹	2000 p ⁹	—

^aEntries: 10 = 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine, 11 = 5,10,15,20-tetrakis(4-sulfonatophenyl)-21*H*,23*H*-porphine, 12 = 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)-21*H*,23*H*-porphine; 13 = 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine, Zn(II); 14 = 5,10,15,20-tetrakis(4-sulfonatophenyl)-21*H*,23*H*-porphine, Zn(II); 15 = 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)-21*H*,23*H*-porphine, Zn(II).

^bn = nonpolar, benzene-like solvent.

^cp = polar solvent.

and 1.00 ± 0.08 in methanol, respectively, and 0.42 ± 0.02 and 0.43 ± 0.02 in water, respectively for λ_{exc} 367 or 437 nm.³¹ Similarly to the bpy complex, these coordination compounds display relatively low singlet oxygen quenching constants by the sensitizer itself ($1.5 \times 10^6 < k_{\Delta, \text{q}}^{\text{PS}} < 1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$).³¹ However, for this type of photosensitizer, the tail of the emission band in the visible part of the spectrum enters the NIR region and may overlap with the $^1\text{O}_2$ emission band centered at 1270 nm. Therefore, corrections for this effect have to be made when these complexes are used as standards for the Φ_{Δ} determination by $^1\text{O}_2$ phosphorescence measurements in solvents such as methanol and specially water, where singlet oxygen signals are extremely weak.³¹ Re(I), Os(II) and Ir(III) complexes can also photogenerate singlet oxygen from their MLCT and LC triplet states, respectively, although with lower quantum yields than Ru(II) complexes, in general.^{36,132} Table 6.5 collects the quantum yields of singlet oxygen production and the photophysical properties of some Ru(II) complexes that have been used as standards for singlet oxygen photogeneration in different solvents.

The photophysical properties of cyclometalated complexes of Ir^{3+} and Pt^{2+} were recently reported and reviewed, with rather high quantum yields of singlet oxygen production in nonpolar solvents.^{133–135} A comparison of the singlet quantum yields of some of these “standard” sensitizers for singlet-oxygen production in various solvents and by different methods is available.¹³¹

6.5.7. Reference Sensitizers for Two-Photon Absorption Experiments

In a two-photon process, a sensitizer excited state, PS_n^* , is produced as a consequence of the simultaneous absorption of two photons of low energy. Even if the state initially populated in a two-photon process is different from that initially populated in a one-photon excitation, it is assumed that rapid relaxation to the electronic singlet state of lowest energy will ensue. In this nonlinear process, the number of sensitizer excited states is generally small and confined to a small sample volume. However, it was established that $^1\text{O}_2(^1\Delta_{\text{g}})$ can be produced by several types of sensitizers.^{136–144} Its detection by time-resolved emission at 1270 nm allowed the determination of the two-photon absorption cross section (δ) of the sensitizer, provided the quantum yield of singlet oxygen production in the one-photon process was accurately known. Ogilby proposed two substituted phenylene vinylenes, 2,5-dibromo-1,4-bis(2-(4-diphenylaminophenyl)vinyl)benzene (BrPhVB, $\Phi_{\Delta} = 0.46$ in toluene and 0.37 in cyclohexane for λ_{exc} 400 nm) and 2,5-dicyano-1,4-bis(2-(4-diphenylaminophenyl)vinyl)benzene (CNPhVB, $\Phi_{\Delta} = 0.11$ in toluene and 0.06 in cyclohexane for λ_{exc} 400 nm) as efficient reference sensitizers for the determination of two-photon absorption cross sections of other molecules in cyclohexane. Actually, singlet oxygen production in neat aromatic solvents precluded their use unless some corrections were made.¹³⁹ Two new aromatic ketones derived from phenalenone, pyrene-1,6-dione (PD) and benzo[*cd*]pyren-5-one (BP), might be suitable alternative standard sensitizers for both one- and two-photon sensitized production of singlet oxygen.

Table 6.5. Quantum yields of singlet oxygen production in air-equilibrated solutions and photophysical properties of some Ru(II) complexes used as standards.^{a,b}

Sensitizer	Solvent	$\Phi_{\Delta} \pm \text{Std dev}$	f_{Δ}^T	$\lambda_{\text{exc}}/\text{nm}$	$E_s/\text{kJ mol}^{-1}$	$E_T/\text{kJ mol}^{-1}$	Φ_T	$\tau_T^0/\mu\text{s}$	$k_q^{O_2}/\text{M}^{-1} \text{s}^{-1}$
16	Methanol	0.73 ± 0.06^{31}	1.0 ± 0.1^{64}	367 or 437	266 p^{31}	197 p^{31}	1.0^{164}	0.72^9	$1.9 \times 10^{9.31}$
	Water	0.22 ± 0.02^{31}	0.48 ± 0.05^{31}	437				0.65^9	$3.3 \times 10^{9.31}$
17	Methanol	0.97 ± 0.08^{31}	1.0 ± 0.1^{31}	367 or 437	259 p^{31}	193 p^{31}	1.0^{164}	3.07^9	$2.4 \times 10^{9.31}$
	Water	0.42 ± 0.02^{31}	0.53 ± 0.05^{31}	437					$3.3 \times 10^{9.31}$
18	Methanol	1.00 ± 0.08^{31}	1.0 ± 0.1^{31}	367 or 437	258 p^{31}	193 p^{31}	1.0^{164}	3.8^{165}	$1.8 \times 10^{9.31}$
	Water	0.43 ± 0.02^{31}	0.55 ± 0.05^{31}	437					$2.9 \times 10^{9.31}$

^aEntries: 16 = tris(2,2'-bipyridyl)ruthenium(II), $[\text{Ru}(\text{bpy})_3]^{2+}$; 17 = tris(4,7-diphenyl-1,10-phenanthrolinyl)ruthenium(II), $[\text{Ru}(\text{dip})_3]^{2+}$; 18 = tris(4,7-diphenyl-sulfonate-1,10-phenanthrolinyl)ruthenium(II), $[\text{Ru}(\text{dpds})_3]^{4+}$.

^bp = polar solvent.

In the one-photon process, these two molecules may be excited at 420 nm and high singlet oxygen quantum yields were shown to be independent on the excitation wavelength in two solvents: for PD, $\Phi_{\Delta} = 0.95 \pm 0.05$ (in toluene) and 1.01 ± 0.05 (in acetonitrile) for λ_{exc} 355 nm and $\Phi_{\Delta} = 0.98 \pm 0.11$ for λ_{exc} 420 nm (in toluene); for BP, $\Phi_{\Delta} = 0.98 \pm 0.05$ (in toluene) and 0.92 ± 0.06 (in acetonitrile) for λ_{exc} 355 nm and $\Phi_{\Delta} = 1.03 \pm 0.12$ for λ_{exc} 420 nm (in toluene).¹⁰⁸ Furthermore, relative to CNPhVB over the wavelength range 650–840 nm, their two-photon absorption cross sections were much larger than that of phenalenone.

6.6. Reference Systems for the Evaluation of Quantum Yields of Singlet Oxygen Production in the Solid Phase

Several recent papers and some reviews have been devoted to the production of singlet oxygen in heterogeneous media, *i.e.*, solid–liquid or solid–gas conditions.^{145,146} Determination of the quantum yields of singlet oxygen production is more difficult in these cases than in homogeneous solutions, mainly because of light scattering by the solid sensitizers (polymers, silica, wool, layered double hydroxides (LDHs), zeolites, *etc.*) and since light absorption by nontransparent samples is hampered, opaque samples are not easily analyzed. Moreover, oxygen diffusion might be limited in such solid media.¹⁴⁷ However, if singlet oxygen quantum yields are not easily determined for these materials, its lifetimes in various media may often be obtained. In the following, instead of classifying the data according to the sensitizer family, they will be presented depending on the type of support.

6.6.1. Sensitizers in Organic Polymers

The first data in this field are related to RB in polymers, pointing out the decrease of the singlet oxygen quantum yield due to RB aggregation in highly loaded polystyrene,^{1,148} and later of acridine in a solid polystyrene sample (cut to the dimension of the fluorescence cuvette and displaying only weak light scattering).¹¹⁴ Interestingly, in this latter case, the singlet oxygen lifetime was found to be of the same order of magnitude in polystyrene (22 μs) and in toluene (29.4 μs), while the singlet oxygen quantum yield decreased from 0.73 in toluene to 0.50–0.58 in the polymer for λ_{exc} 355 nm.

More recently, a detailed photophysical study of methylene blue (MB) in Nafion films was carried out in order to propose a readily available reference photosensitizing *transparent* material, starting from commercial components.¹⁴⁹ It was shown that MB was located in a nonpolar environment in air dried MB–Nafion films. From the comparison of the decay of the MB triplet excited state and of $^1\text{O}_2$ production (by phosphorescence emission at 1270 nm with reference to $[\text{Ru}(\text{bpy})_3]^{2+}$ in acetonitrile solution), it was concluded that all the triplet states were quenched by O_2 both in solution and in Nafion, and that the decreased singlet oxygen quantum yield in Nafion ($\Phi_{\Delta} = 0.24\text{--}0.35$ for

λ_{exc} 532 nm) was assignable to a decreased triplet quantum yield. In methanol swollen MB-Nafion films, the results closer to those in MeOH ($\Phi_{\Delta} = 0.47$) indicated that $^1\text{O}_2$ was produced in a methanolic environment. The long singlet oxygen lifetimes (85–90 μs) in Nafion were noticeable and longer than in water or methanol-equilibrated MB-Nafion films. Other materials, based on C_{60} fullerene derivatives or Ru(II) polypyridyls embedded in porous silicone, also produce singlet oxygen with lifetimes in the range 40–47 μs , with strong aggregation effects especially in the case of the materials containing C_{60} . Therefore, these materials cannot be considered as references in the solid phase.⁸³

In a continuous effort to develop antibacterial polyurethane fibers (110–250 nm) and nanofabrics loaded with porphyrin or phthalocyanine sensitizers, singlet oxygen-sensitized delayed fluorescence (SODF) was used in addition to more conventional spectroscopy for a sensitive time-resolved detection of $^1\text{O}_2$, especially at low concentrations, where the direct measurement is difficult due to the extremely low quantum yield of its luminescence at 1270 nm.¹⁵⁰ Very recently, electrospun polystyrene nanofibers modified with an externally bound cationic porphyrin photosensitizer were tested for photo-oxidation in aqueous media. They were shown to be as efficient as polystyrene nanofibers with embedded porphyrin, despite a shorter singlet oxygen lifetime (0.7 and 13.5 μs , respectively), due to the necessary diffusion of $^1\text{O}_2$ to the surface of the fiber in the case of embedded sensitizer.¹⁴⁷ The crucial role played by surface hydrophilicity/wettability in achieving efficient photo-oxidation of polar compounds adsorbed at the surface of a material generating $^1\text{O}_2$ with a short diffusion length (205 nm) from the fiber to its surface was demonstrated.¹⁵¹

6.6.2. Sensitizers in Silica or Glass

Singlet oxygen was also efficiently generated in aqueous solution by a cationic porphine dye adsorbed on porous Vycor glass (PVG),^{152,153} or by a silicon phthalocyanine covalently bound to glass prepared by a sol method,¹⁵⁴ providing a heterogeneous system for use in water (no quantitative data on singlet oxygen production due to the lack of a reference PVG sensitizer).

Lifetimes of singlet oxygen produced by various sensitizers embedded in silica monoliths and comparison of their singlet oxygen quantum yield relative to phenalenone in the same material could be carried out by time-resolved and steady-state phosphorescence at 1270 nm thanks to the high transparency of the samples that were directly inserted in the spectroscopy setup.¹⁵⁵ Singlet oxygen lifetimes in these hydrated silica samples were in the range 17–25 μs , *i.e.* much longer than in methanol or water (9.5 and 3.5 μs , respectively). High singlet oxygen quantum yields (0.9–1.0) relative to phenalenone in the same silica materials were determined for 9,10-dicyanoanthracene or for the recently synthesized cyanoaromatic benzo[*b*]triphenylene-9,14-dicarbonitrile (DBTP).¹⁵⁵ Other phenothiazines, cyanoaromatics or quinones embedded in silica monoliths were compared and the singlet-oxygen quantum yields correlated with solvent-free oxidation of gaseous dimethylsulfide.^{156,157}

A large number of studies are currently devoted to the preparation of silica nanoparticles with embedded singlet oxygen photosensitizers, mainly for photodynamic therapy (PDT) applications. In most cases, singlet oxygen quantum yields are determined by conventional methods using nanoparticle suspensions in suitable solvents. To the best of our knowledge, no specific standard was proposed in these cases.

6.6.3. Sensitizers in Zeolites

Although the production of singlet oxygen by sensitizers encapsulated in zeolites is well documented, quantitative data on the quantum yields of singlet oxygen production in these systems are scarce, probably due to the difficult determination of light absorption in these highly scattering media.¹⁴⁵ However, singlet oxygen lifetimes in zeolites were determined to be in the range between 7.5 and 7.9 μs .^{158,159} More recently, fluorescence and singlet oxygen quantum yields of a Si(IV)-phthalocyanine bound to the surface of zeolite L nanocrystals ($\Phi_{\Delta} = 0.40$ for λ_{exc} 680 nm) were compared with the same sensitizer in homogeneous dichloromethane solution ($\Phi_{\Delta} = 0.50$ for λ_{exc} 680 nm) due to the fact that these nanomaterials are readily dispersed in this solvent. For aqueous suspensions of these nanomaterials ($\Phi_{\Delta} = 0.35$ for λ_{exc} 680 nm), the absorption spectra had to be baseline corrected due to light scattering.¹⁶⁰

6.7. Conclusions

Photosensitization is the preferred method of singlet molecular oxygen ($^1\text{O}_2$) generation. Synthetic, environmental and biomedical applications of this metastable species can be easily developed by using photosensitizing dyes with intense UV-vis light absorption and excited states that can transfer their excitation energy to ground-state molecular oxygen. The quantum yield of singlet oxygen production (Φ_{Δ}) represents the amount of photogenerated $^1\text{O}_2$ molecules per amount of photons absorbed by the photosensitizer. Therefore, Φ_{Δ} is a key parameter that has to be accurately known before the development of any singlet oxygen application. Various organic or metal-organic dyes have the ability to produce singlet oxygen and several methods of Φ_{Δ} determination have been developed. Most of these methods, such as $^1\text{O}_2$ scavenging by a chemical probe or $^1\text{O}_2$ phosphorescence in the near-infrared, make use of relative measurements where the experimental results of the studied photosensitizing system are compared with those of a standard or reference sensitizer. For this reason, there is a need for singlet oxygen sensitizers with precisely known Φ_{Δ} values under defined experimental conditions that can be used as references for Φ_{Δ} determination of new $^1\text{O}_2$ photosensitizing dyes or systems.

Both intrinsic and extrinsic factors can influence singlet oxygen photogeneration. The former are related to the nature and structure of the

sensitizer itself, whereas the latter are dependent on the physical conditions and chemical environment around the sensitizer. Some of the intrinsic and/or extrinsic factors can be interdependent and, therefore, it is useful to know to what extent these complex interdependencies can affect singlet oxygen production.

All these factors have been thoroughly reviewed in this chapter, as well as the constants and data usually used to describe singlet oxygen production by sensitization. The intrinsic factors allow distinguishing several types of sensitizers, namely ST, TC or T types, depending on the properties of their excited singlet (S) and triplet (T) states. Generally, triplet (T) sensitizers with high intersystem crossing quantum yields, long-lived triplet excited states (tens of μs), and with E_{T1} larger than E_{A} (energy of the lowest singlet excited state of molecular oxygen $^1\Delta_{\text{g}}$, $E_{\text{A}} = 94 \text{ kJ mol}^{-1}$) are able to produce singlet oxygen only from their triplet state. They show Φ_{A} values that are less dependent on the oxygen concentration than those of the other types of sensitizers (ST or TC), even though the rate constant of the bimolecular quenching of the triplet state by O_2 ($k_{\text{T},\text{q}}^{\text{O}_2}$) is typically one order of magnitude lower than the diffusion-controlled limit. From the numerous literature data, the very complex competition between energy- and charge-transfer processes following quenching of the excited sensitizer by ground-state O_2 is also shown to strongly influence Φ_{A} . Regarding the extrinsic factors, oxygen concentration and solvent viscosity and polarity are the most important experimental variables that have to be considered.

Accordingly, the most commonly used reference $^1\text{O}_2$ sensitizers are photo-stable T-type sensitizers, easy to prepare or commercially available dyes, with intense absorption bands in the UV-vis region, high Φ_{A} values in a wide variety of solvents, and with a low quenching rate constant of the photogenerated singlet oxygen. 1*H*-Phenalen-1-one (also known as perinaphthenone or phenalenone) is an aromatic ketone soluble in polar protic and aprotic solvents as well as in nonpolar media, which can be considered as the universal or “gold” standard ($\Phi_{\text{A}} = 0.95 \pm 0.05$) for singlet oxygen production and quantitative evaluation in the liquid phase. Other aromatic ketones and quinones such as benzanthrone, 4-phenylbenzophenone and 2-acetonaphthone have been proposed as standards for $^1\text{O}_2$ production as well. Besides the aromatic ketones, other sensitizers have also been used as references, not only in liquid but also in the solid phase. Rose bengal, methylene blue and acridine heterocycles, and the C_{60} fullerene carbon nanoform are well-characterized $^1\text{O}_2$ photosensitizers, with high Φ_{A} values, that can be used in solvents of different polarity and protic character, and easily immobilized onto distinct polymer supports. 5,10,15,20-Tetraphenyl-21*H*,23*H*-porphine and its ionic (tetrasulfonated and tetramethylpyridinium) derivatives and Zn(II) complexes are a family of porphyrin sensitizers that have been commonly used as references in photodynamic therapy studies. Several Ru(II) coordination compounds with polyazaheterocyclic ligands have also been thoroughly characterized as $^1\text{O}_2$ photosensitizers in different solvents and their use has been reported for a wide variety of $^1\text{O}_2$ applications in the liquid and solid phases.

Finally, it should be stressed that for an accurate singlet oxygen quantum yield determination of any novel photosensitizer or of a known sensitizer under new conditions, a previous careful examination of several parameters of the system under study (nature of the sensitizer excited state, concentration effects, excitation wavelength, temperature, oxygen concentration, solvent) and of the properties of the standard sensitizer under the same conditions has to be done. A deep understanding of the quenching processes of the sensitizer excited states and of singlet oxygen is always a prerequisite. These numerous requirements may explain why in most publications dealing with photosensitized production of singlet molecular oxygen, only relative values (kinetic curves or relative singlet oxygen quantum yields) are given. These determinations are still more complicated when using solid-phase sensitizers because the photophysical properties may be different, diffusion of reactants is limited and light-scattering effects are difficult to avoid. This chapter aims at helping photochemists with the design of experiments for the photosensitized production of singlet oxygen, and proposes a set of reference compounds or materials that can be trusted and may be useful for an accurate evaluation of singlet molecular oxygen production in solution or in the solid phase.

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Chapter 7

The Sensitized Production of Singlet Oxygen Using Two-Photon Excitation

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7.1. Introduction

The use of two simultaneously absorbed photons to create an excited electronic state was first experimentally realized in the early 1960s with the advent of the laser, simply because this tool could deliver the required high radiant intensity of photons.¹ Thereafter, an appreciable effort was initiated to examine fundamental aspects of two-photon spectroscopy (*e.g.*, what states are populated in a two-photon transition as opposed to a one-photon transition).^{2–4} More recently, the field has seen a resurgence due principally to (a) the advent of femtosecond lasers,⁵ (b) the systematic development and synthesis of chromophores that have very large two-photon transition probabilities,⁶ and (c) the use of two-photon excitation to create spatially localized excited-state populations (*i.e.*, the development of two-photon-based microscopes).⁷ This activity has had significant ramifications in disciplines that range from polymer science^{8,9} to biological imaging.^{10,11} A number of relatively recent reviews and books summarize selected aspects of the field.^{5,12–16}

Over the past ~15 years, we have explored and exploited issues related to the two-photon excitation of a singlet oxygen sensitizer.¹⁷ The topics addressed range from the fundamental photophysics of such sensitizers^{18–25} to the use of this excitation as a mechanistic tool to better elucidate the behavior of singlet oxygen in single-cell experiments.^{26–30} The material in this chapter summarizes some of our efforts in this regard.

7.2. Femtosecond Lasers

It is advantageous at the outset to briefly elaborate on the statement made above regarding femtosecond lasers. In the context of exciting two-photon transitions, the intent here is principally to examine the advantages/disadvantages of a laser that delivers fs pulses (*e.g.*, Gaussian full width at half-maximum, FWHM, ~100 fs) as opposed to a laser that delivers ns pulses (FWHM ~ 4–8 ns).

The principal advantage of using a fs laser is that the molecule under study is exposed to a high *irradiance*. The units of irradiance are W m^{-2} . Noting that the unit of power (a watt, W) is equivalent to J s^{-1} , it should be clear that by shortening the time over which photons are delivered to the molecule better facilitates the high density of photons required for the two-photon process. Another advantage of using a fs laser, certainly at the limit where higher irradiances are used, is that one minimizes the extent to which the excited states thus produced can also absorb a photon from the exciting pulse. For example, and as discussed further in the next section with respect to Figure 7.1, the time required for intersystem crossing and the production of a triplet state is generally sufficiently long relative to the fs pulse duration. Thus, absorption of the incident light by the molecule's triplet state does not compete with light absorption by the molecule's ground state. This is very important in terms of accurately quantifying the two-photon transition probability of the ground state to produce an excited state (*i.e.*, the so-called two-photon absorption cross section).

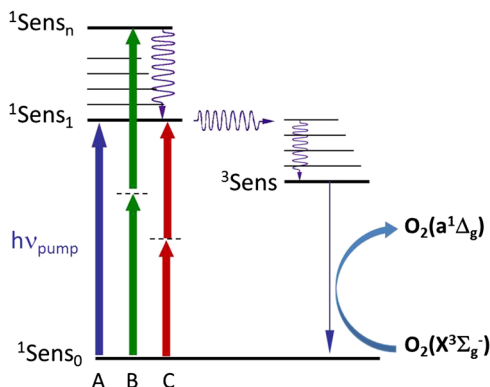


Figure 7.1. Diagram illustrating several aspects pertinent to the two-photon excitation of a singlet oxygen sensitizer. It is assumed that the sensitizer ground state is a singlet spin state, $^1\text{Sens}_0$. (A) Illustration of a one-photon process that populates the lowest excited electronic state, $^1\text{Sens}_1$, of the sensitizer. (B and C) Illustrations of two-photon processes by which a sensitizer excited state can be populated. To illustrate how selection rules can have an effect, the state populated in (B) is different from that populated in the one-photon transition, whereas in (C) the state produced in the two-photon transition is the same as that produced in the one-photon transition. Both two-photon processes are shown to proceed through a virtual state represented by the dashed line.

The points mentioned above provide sufficient justification for the often-repeated statement that lasers delivering fs pulses “must” be used in two-photon experiments, certainly those that involve some parameter that needs to be quantified or controlled. However, there are also distinct disadvantages or complications that come with using fs pulses (other than the expense associated with acquiring such a laser). First, it is well established that a two-photon transition is very sensitive to the characteristics of the excitation source.^{5,14,31} Thus, in quantifying two-photon absorption cross sections, temporal and spatial coherence factors of the incident fs pulse must be included in the analysis.⁵ These corrections must reflect pulse parameters at the sample to account for common optics-related perturbations of a fs pulse (*i.e.*, the pulse can be “chirped”). Secondly, one must always recognize that, as a consequence of Heisenberg’s uncertainty principle, a decrease in the time duration of the laser pulse is accompanied by a corresponding increase in the spectral bandwidth of the pulse (*e.g.*, ~10–20 nm for typical fs pulses). This is certainly different from the output of a ns laser, which is effectively monochromatic.

7.3. Creating the Excited State: Spectral Selectivity

Pertinent aspects of two-photon excitation for a generic organic molecule are illustrated in Figure 7.1. The intent is to show a process in which the ground electronic state of the molecule interacts with a photon to produce a so-called virtual state that, in turn, interacts with a second photon to produce

a discrete excited electronic state. The virtual state represents a nonresonant situation in which the electron distribution of the molecule is responding to the electromagnetic perturbation of the incident light. A resonant transition in which energy is absorbed from the incident radiation field can only be achieved upon interaction of this extremely short-lived virtual state with a second photon that is present under conditions of high irradiance.

The first and most obvious point to be made is that, for a transition to populate a given state, the irradiation wavelength required for the two-photon process is invariably longer than that required for the corresponding one-photon process. Of course, this simply reflects the fact that, in the two-photon process, it is the combined energy of the individual photons that is used to realize the transition.

Carrying the latter point further, recall that one-photon electronic transition energies for typical polyatomic organic chromophores correspond to light over the wavelength range $\sim 300\text{--}700$ nm. Thus, the photon energies at wavelengths over the range $\sim 700\text{--}900$ nm, which are typically used in a two-photon experiment, are generally not sufficient to populate the lowest excited electronic state of many organic chromophores in a one-photon process. This means that one can use the longer wavelengths associated with two-photon processes to impart selectivity in the excited state produced upon irradiation of a complex system containing many chromophores. For example, in a mammalian cell, by introducing a molecule that has a two-photon absorption cross section greater than that of endogenous molecules, one can potentially avoid unwanted photoinitiated processes associated with using a shorter wavelength of light that is readily absorbed by many molecules in a one-photon process. In short, one can potentially achieve much greater selectivity in the excited states created using two-photon excitation.

The selection rules for a given transition in a given molecule may dictate that the state initially populated in the two-photon process is different from that populated in a one-photon process.² This phenomenon is also illustrated in Figure 7.1. Examples of representative spectra are given in Figure 7.2. Irrespective of what excited state is initially populated, however, Kasha's rule should nevertheless still be obeyed. Thus, with few exceptions, the same fluorescent state will be formed irrespective of the mode of excitation. Thereafter, for molecules specifically chosen for use as singlet oxygen sensitizers, intersystem crossing will occur with the same efficiency to yield the more effective singlet oxygen precursor, the longer-lived triplet state.

7.4. The Photophysics of Two-Photon Singlet Oxygen Sensitizers: The Great Compromise

It should be apparent from Figure 7.1 that, in the ideal case, a desirable two-photon singlet oxygen sensitizer will have the combination of a large two-photon absorption cross section and a high yield of a long-lived triplet state that efficiently produces singlet oxygen upon interaction with

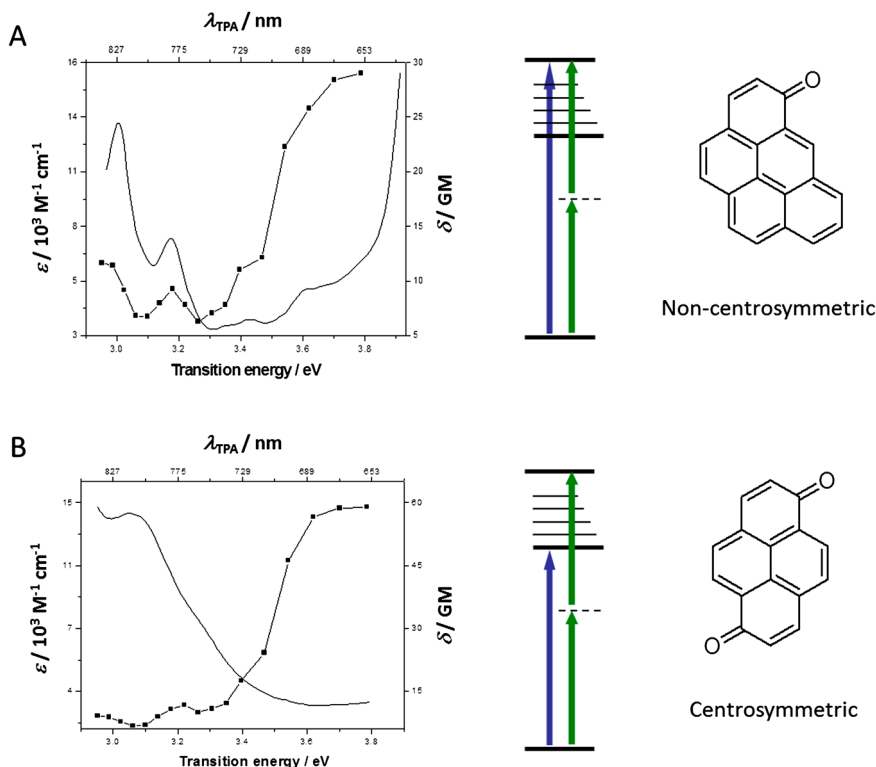


Figure 7.2. Examples of one-photon absorption (solid lines, left side ordinate) and two-photon excitation (symbols, right side ordinate) spectra for molecules in which the respective processes produce (A) the same excited state, and (B) different excited states. For these examples, taken from our own work on two-photon singlet oxygen sensitizers, the pertinent feature that distinguishes one process from the other is whether or not the ground state of the molecule has a center of inversion.²³ The wavelength scales shown on the upper x-axes refer only to the two-photon spectra, whereas the total transition energy shown on the bottom x-axes refers equally to both the one- and two-photon spectra.

ground-state oxygen. Unfortunately, however, the general factors that give rise to large two-photon absorption cross sections (*e.g.*, intramolecular separation of charge that yields a large transition dipole moment^{6,12}) are generally not conducive to the production of singlet oxygen.¹⁷ Specifically, molecules that facilitate intramolecular charge separation also generally form low-energy charge-transfer, CT, complexes with oxygen. The latter mediate non-radiative processes of excited-state deactivation that kinetically compete with energy transfer to produce singlet oxygen.^{32–36} Hence, the “great compromise”; find a molecule that has just enough CT character to yield an acceptably large two-photon cross section, but not so much CT character as to adversely influence the yield of singlet oxygen. Of course, the extent to

which CT character plays a role in the photophysical processes that characterize interactions between oxygen and organic molecules depends not just on the molecule in question (*e.g.*, its oxidation potential), but also on the solvent or local environment around the chromophore.³²

With the “great compromise” in mind, good singlet oxygen sensitizers can nevertheless still have sufficiently large two-photon absorption cross sections to make them useful in many applications. As illustrated in Table 7.1, it is possible to have a two-photon absorption cross section as large as ~ 1000 GM (where the unit of 1 Göppert-Mayer, GM, is equal to 10^{-50} cm⁴ s photon⁻¹) with a singlet oxygen quantum yield, measured in a one-photon experiment, of ~ 0.3 – 0.5 . As with many things, exceptions exist. For example, an alkyne-linked porphyrin dyad has been produced that has a remarkably large two-photon absorption cross section of $\sim 17\,000$ GM and a one-photon singlet oxygen quantum yield >0.3 , the latter depending on the solvent used (Table 7.1).³⁷

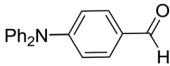
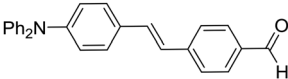
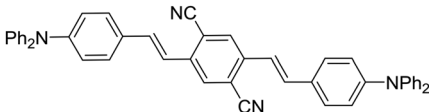
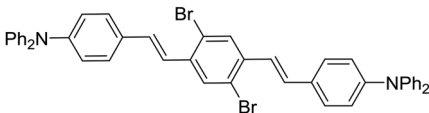
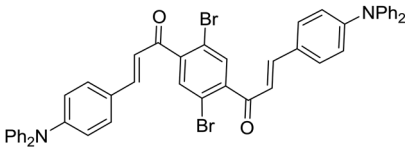
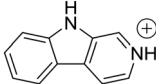
Although attempts to maximize the combination of the two-photon absorption cross section and the singlet oxygen yield are reasonable, care must be exercised not to overestimate the importance of the absorption cross section. For many applications, it is sufficient just to increase the incident laser irradiance to produce a higher concentration of excited states *via* two-photon absorption. This approach is acceptable because, at the wavelengths involved, many systems will simply transmit the light through the sample if it is not absorbed *via* the two-photon electronic transition. Of course, to avoid sample heating, care must be taken to ensure that solvent vibrational overtone transitions play a sufficiently small role at the irradiation wavelength. In short, in the search for a suitable two-photon sensitizer, it is generally more prudent to focus on finding a molecule that has a large yield of singlet oxygen production rather than a molecule with a large two-photon absorption cross section.

A different approach to the problem of the “great compromise” is to use a two-component system consisting of an efficient two-photon light-absorbing chromophore coupled to a separate singlet oxygen sensitizer.⁴⁴ In a successfully designed system, energy transfer from the light-absorbing chromophore to the sensitizer ensues, thus generating singlet oxygen in high yield.

7.5. The Two-Photon Sensitized Production of Singlet Oxygen as a Photophysical Tool

Recording the two-photon absorption spectrum and quantifying the associated absorption cross sections of a molecule are important for the complete photophysical characterization of a two-photon singlet oxygen sensitizer (*e.g.*, data in Figure 7.2). These are nontrivial exercises, however, and are susceptible to technique-related errors that can yield inaccurate results.^{5,14,21,31} It is important to keep this caveat in mind, when considering the many ways by which these data can be obtained.

Table 7.1. Two-photon absorption cross sections, δ , and singlet oxygen quantum yields, Φ_{Δ} , for selected molecules in air-saturated solutions.

Molecule	Solvent	δ^a (GM)	Φ_{Δ}	Ref.
	Toluene	59 ± 12 @ 700 nm	0.25 ± 0.02	38
	Toluene	180 ± 36 @ 800 nm	0.04 ± 0.01	38
	Toluene	2815 ± 420 @ 845 nm	0.11 ± 0.02	21
	Toluene	1310 ± 200 @ 780 nm	0.46 ± 0.05	21
	Toluene	1740 ± 260 @ 845 nm	0.28 ± 0.03	24
	H ₂ O pH 4.8	1.35 ± 0.27 @ 600 nm	0.10 ± 0.01	39
1-Phenalenone	Toluene	6.5 ± 1.0 @ 685 nm	0.97 ± 0.06	23
Tetraphenylporphyrin	Toluene	24 @ 760 nm	0.66 ± 0.08	40 and 42
2,7,12,17-Tetraphenylporphycene	Toluene	2280 ± 350 @ 770 nm	0.23 ± 0.02	22
Protoporphyrin IX	Ethanol	2 @ 790 nm	0.56	41 and 42
A dendrimer-encased Pd porphyrin	PBD ^b	27.9 ± 4.2 @ 800 nm	0.88 ± 0.06	28
An alkynyl-linked Zn porphyrin dimer	DMF	17 000 @ 916 nm	0.60 ± 0.06^c 0.25 ± 0.03^d	37
Flavin mononucleotide (FMN)	PBD	3.4 ± 0.4 @ 722 nm	0.65 ± 0.04	25 and 43
A protein-encased FMN ("miniSOG")	PBD	3.7 ± 0.7 @ 722 nm	0.030 ± 0.002^e	25 and 43

^aThe wavelength at which this cross section was determined is also shown. Generally, this corresponds to the maximum of an absorption band.

^bPBD = phosphate-buffered D₂O.

^cRecorded in methanol.

^dRecorded in D₂O.

^eThe reason for such a low quantum yield in this case depends more on the local environment than on the chromophore itself; electron transfer from the protein to FMN kinetically competes with singlet oxygen production.⁴³

The most common approach to quantify two-photon absorption properties relies on using the intensity of a given molecule's fluorescence as a measure of the number of excited states produced upon two-photon excitation.⁵ Nevertheless, this approach also has its limitations; in the least, the molecule must have an appreciable quantum yield of fluorescence. Thus, it is generally desirable to have access to complementary tools that can be used according to the stipulations of a given experiment.^{21,23}

For molecules that sensitize the production of singlet oxygen, the intensity of the ~ 1275 nm $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence thus produced can likewise be used to quantify the number of excited states created upon two-photon irradiation of the sensitizer.^{21,23} The advantages of this approach include the facts that (a) singlet oxygen phosphorescence at ~ 1275 nm is invariably sufficiently red-shifted with respect to transitions in the molecule under study that, in turn, minimizes complications in the detection protocol, (b) the emission spectrum of singlet oxygen is a constant that is independent of the molecule used as a sensitizer, (c) the singlet oxygen lifetime is generally in the microsecond domain, and this facilitates temporal discrimination against common pulsed-laser-dependent artifacts in the nano- and femto-second domains, and (d) when comparative experiments performed against a given two-photon standard call for a change in solvent, all of the pertinent information regarding solvent-dependent changes in the radiative lifetime of singlet oxygen are available.^{33,45}

The two-photon singlet oxygen experiments used to quantify sensitizer absorption cross sections are readily performed using 1 cm path length cuvettes where (a) the number of excited states created is relatively large, and (b) where it is easier to detect emission from these excited states.

7.6. Creating the Excited State: Spatial Selectivity

Because the two-photon absorption process only occurs where the incident irradiance is sufficiently high, the use of a focused laser as the irradiation source can result in a three-dimensional spatially localized population of excited-state sensitizers that, in turn, can yield a spatially localized population of singlet oxygen.²⁶ However, for microscope-based experiments performed on single cells, which lie "flat" on a cover slip, the biggest advantage in this regard is that incident light scattered by the cell is not sufficiently intense to excite a two-photon transition. Thus, relative to the corresponding process of one-photon excitation, one gains appreciable lateral spatial resolution with respect to the excited states produced (Figure 7.3).²⁷⁻²⁹

Given that the production of excited states *via* two-photon excitation depends on the irradiance of the incident laser beam, the probability with which a two-photon transition can be achieved will only be sufficiently large in a small portion of a diffraction-limited focused beam. As such, it is possible to achieve sensitizer excitation with a spatial resolution that exceeds the dimensions determined directly by the diffraction of light.^{26,46} This point

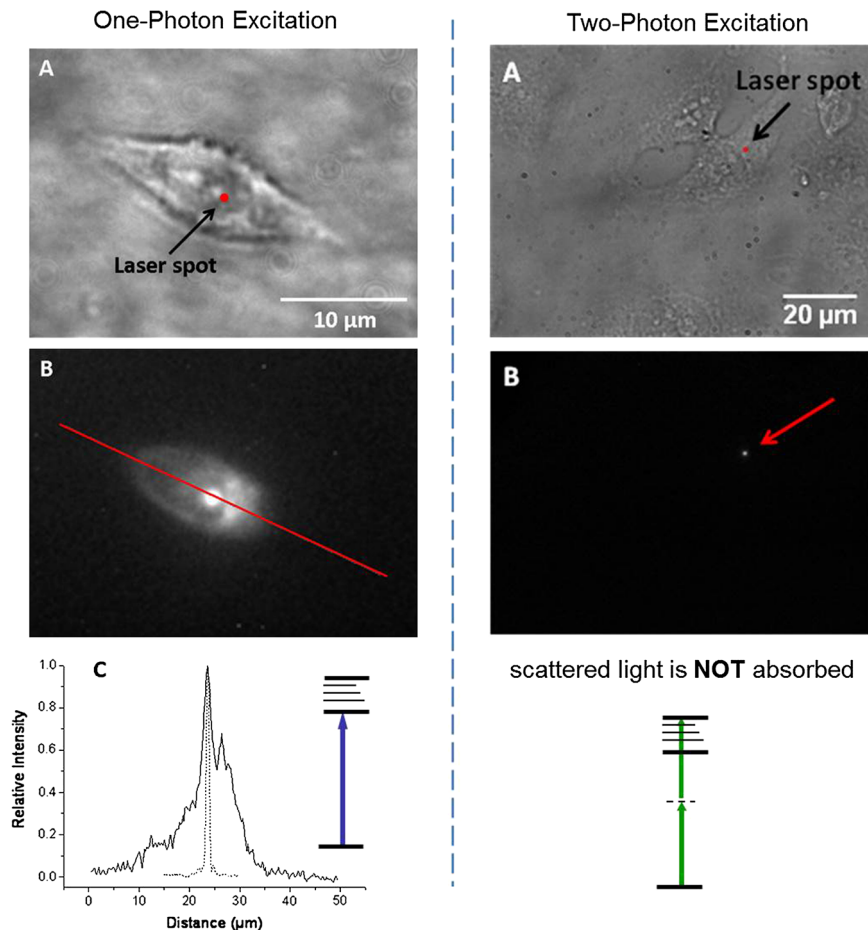


Figure 7.3. Images that illustrate how two-photon excitation (right-hand side) of an intracellular sensitizer facilitates an increase in the lateral spatial resolution in a single-cell experiment, as compared to one-photon excitation (left-hand side). In both cases, panels A show bright-field images of the cells with a superimposed spot that approximates the $\sim 1\ \mu\text{m}$ diameter cross section of the focused laser beam used to irradiate the sensitizer (intracellular protoporphyrin IX, PpIX). Panels B show the resultant PpIX fluorescence. Panel C on the left-hand side shows the fluorescence intensity along the transcellular line drawn in panel B. The dashed line in panel C represents the spatial distribution of the exciting laser pulse. The one-photon data reflect the fact that incident light scattered by the cell is easily absorbed by PpIX throughout the cell. This figure is a modified form of data originally published in ref. 27.

is illustrated in Figure 7.4 where a diffraction-limited lateral resolution of $\sim 1000\ \text{nm}$ (*i.e.*, cross-sectional spot size) is shown for light with a wavelength, λ , of $800\ \text{nm}$ focused with a microscope objective whose numerical aperture, NA, is 0.9. However, one must not overlook the fact that, once formed by a spatially confined population of excited-state sensitizers, singlet oxygen can diffuse over an appreciable distance given that its lifetime is generally quite

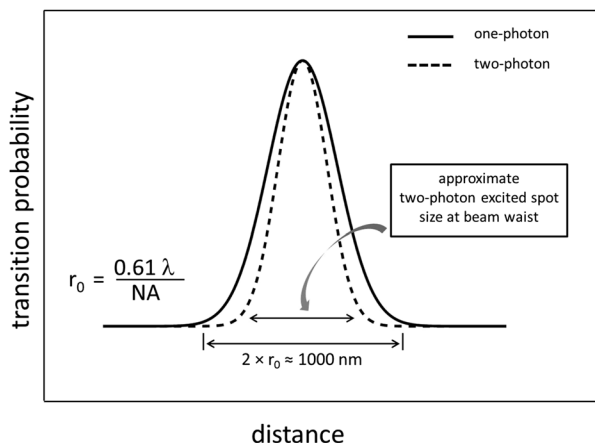


Figure 7.4. Cartoon that illustrates aspects of two-photon excitation related to the spatially dependent intensity profile of the laser pulse at the sample (*e.g.*, at the focal plane on a microscope stage). The solid line represents the spatially dependent probability for a one-photon transition, using a Gaussian profile to approximate the intensity profile of the focused incident light. The diffraction-limited cross-sectional diameter (*i.e.*, spot size = $2 \times r_0$) of $\sim 1000 \text{ nm}$ was calculated for a wavelength, λ , of 800 nm and a microscope objective with a numerical aperture, NA, of 0.9 . The dashed line represents the corresponding probability for a two-photon transition, obtained by taking the square of the one-photon transition probability. Given the dependence of two-photon excitation on the square of the incident light intensity, the spot size of excited states created can be smaller than that limited by the diffraction of light (*i.e.*, the spot size that would be observed in a corresponding process of one-photon excitation).

long and, as a small molecule, its diffusion coefficient is generally large. For example, in an H_2O -incubated cell, where we assume a value of $4 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for the diffusion coefficient of oxygen⁴⁷ and where the singlet oxygen lifetime is $\sim 3 \mu\text{s}$,^{17,48} the radial diffusion distance of singlet oxygen over a period of $9 \mu\text{s}$ (*i.e.*, ~ 3 lifetimes) is $\sim 150 \text{ nm}$.^{17,47}

We have exploited this ability to create small, localized populations of singlet oxygen in a range of cell-based experiments.^{27–30} For example, one can use such focused irradiation to selectively create singlet oxygen in one cell, at the exclusion of creating singlet oxygen in a neighboring cell. In this way, one has a useful tool to examine aspects of the so-called “bystander effect” where processes occurring in one cell can influence those in a nearby cell.²⁹ Carrying this approach further of selectively exciting one cell at the exclusion of exciting a neighboring cell, we have also shown that the controlled production of a low dose of singlet oxygen in one cell can cause it to enter the mitotic cycle much earlier than a neighboring sister cell that had not been irradiated.³⁰ This dose-dependent stimulatory response provides a nice contrast to the commonly observed cytotoxic effects of singlet oxygen and, thereby, facilitates further studies into the different roles that singlet oxygen plays in mechanisms of cell signaling. Finally, we have also used such localized two-photon irradiation

to create extracellular populations of singlet oxygen adjacent to the cell membrane.²⁸ In this way, when compared to the more traditional intracellular production of singlet oxygen, we provide an interesting approach to change the initial targets of singlet oxygen action. The latter can have significant mechanistic ramifications with respect to elucidating the roles played by singlet oxygen in cell signaling and, at the limit, cell death.

It is sometimes mentioned that because two-photon transitions of singlet-oxygen sensitizers occur at wavelengths where tissue is most transparent (*i.e.*, $\sim 700\text{--}900\text{ nm}$), one could achieve much greater depth penetration with the treatment methodology of photodynamic therapy, PDT, simply by using a focused laser to sensitize the production of singlet oxygen in a two-photon process. The caveat here, however, likewise draws upon the effects of light scattering; because of scattering by the tissue, the light may not be sufficiently intense to create the required cytotoxic population of singlet oxygen. Indeed, the process under these conditions may even be counterproductive in that, because of light scattering, the two-photon-sensitized dose of singlet oxygen may be low enough to stimulate rather than kill cells.³⁰ It is nevertheless important to note that remarkable tissue depth penetration has been achieved in two-photon promoted tumor regressions⁴⁹ and fluorescence imaging experiments.^{50,51}

7.7. Detecting Singlet Oxygen Phosphorescence in Spatially Resolved Two-Photon Sensitized Experiments

Although the $\sim 1275\text{ nm}$ time-resolved phosphorescence of singlet oxygen has thus far been detected from a wide range of systems, one must not lose sight of the fact that this phosphorescence is inherently very weak.^{33,45,52}

In the present context, we note that singlet oxygen phosphorescence has indeed been detected in a variety of two-photon-sensitized experiments, even though the absolute number of excited states produced is invariably less than that produced in the corresponding one-photon process.^{17,26} However, for many systems of current research relevance (*e.g.*, single cells,^{48,53,54} thin polymer films^{55,56}), the singlet oxygen detected by its phosphorescence has, to the author's knowledge, always been produced through the more efficient one-photon-sensitized process that results in a much larger absolute number of excited states created.

In aqueous biological systems that contain singlet oxygen quenchers (*e.g.*, proteins), the quantum efficiency of singlet oxygen phosphorescence is generally in the range $\sim 10^{-7}\text{--}10^{-9}$. Thus, in a microscope-based spatially resolved two-photon sensitized experiment performed on a living cell, the absolute number of photons emitted by the singlet oxygen molecules created is small.²⁶ To the author's knowledge, no one has, thus far, been able to detect singlet oxygen phosphorescence in single-cell experiments upon focused two-photon excitation of an intracellular sensitizer under conditions that are mechanistically useful.²⁶ It is important to note that, under these latter

conditions of focused two-photon excitation of a sensitizer, the amount of singlet oxygen thus produced is still large enough to be cytotoxic and the morphological changes associated with cell death are readily observed.^{27,29,54}

7.8. Spatial Localization in and on Cells: Solvent Effects and the Effects of Protein Enclosures on Genetically Encoded Sensitizers

For biologically relevant studies, an effective way to localize a chromophore at a specific place in or on a cell is to associate the pertinent molecule with a protein and then to use the techniques of genetic engineering to localize that chromophore-bearing protein at the desired cellular location.^{57,58} Because cells are inherently inhomogeneous, the local environment of the chromophore could thus vary appreciably as a function of where in or on a cell it is placed. In turn, these changes in the local environment could influence selected photophysical properties in different ways (*e.g.*, change yields of fluorescence and/or singlet oxygen). Therefore, it could be advantageous not to just “associate” the desired chromophore with a protein but rather to encapsulate the chromophore with the protein. In this way, irrespective of where the chromophore-bearing protein is placed, the local environment surrounding the chromophore should always be the same. With this perspective in mind, it becomes necessary to ascertain to what extent two-photon absorption spectra and cross sections depend on the surrounding environment and, in particular, on the presence of an encapsulating protein. Clearly, this is a key step in quantifying, and perhaps more importantly, gaining control over parameters that influence the production of singlet oxygen.

It is first useful to consider data obtained from chromophores dissolved in a bulk solvent. To the author’s knowledge, there are not many studies that have examined how a change in solvent can/will influence two-photon absorption. In the least, we have shown that it may be difficult to establish general rules about how a given solvent change may be manifested.⁵⁹ Specifically, we have demonstrated that the solvent effects that have been observed depend more on the solute used rather than the solvents involved (*e.g.*, a change from acetonitrile to cyclohexane can cause an increase in the absorption cross section for one solute, but causes a decrease in the cross section for a related and very similar solute).⁵⁹ Although the solvent-dependent changes observed in this particular study were not large, they nevertheless are large enough to cause problems if one is concerned with controlling doses of sensitized singlet oxygen production. The current conclusion is that, if a change in solvent is anticipated for a given study, the proper control experiments must be performed to characterize the two-photon properties of each sensitizer in these particular solvents.

This same line of reasoning is applicable when considering the effect that a given protein enclosure can have on the two-photon properties of a chromophore. Drobizhev *et al.*¹⁶ have recently shown that, for a series of the “fruit”

fluorescent proteins (*e.g.*, mTangerine, mStrawberry, mPlum, and tdTomato), all of which have the same chromophore, changes in the local protein environment can have pronounced effects on the two-photon absorption cross section. This response was attributed to protein-dependent changes in the local electric field at the chromophore.¹⁶ On the other hand, we recently examined the behavior of flavin mononucleotide (FMN) dissolved in bulk water and, independently, encased in a protein.²⁵ For this case, the change in local environment has an appreciable effect on the lifetime of the FMN triplet state and on the yield of FMN sensitized singlet oxygen. However, we found that this change in local environment had no effect on the two-photon absorption spectrum and cross sections. In an attempt to better understand our results, we computationally modeled how these respective environments influenced the electrostatic potential across the chromophore and found that, indeed, in both cases, the potential at each point in the molecule was the same. Despite the inference that such computations might obviate the need for control experiments on related systems, we believe the field is still young enough to demand more experimental data.

7.9. Conclusions

The one-photon photosensitized production of singlet oxygen is a “mature field”. It has been studied and exploited extensively for the past ~50 years. The two-photon photosensitized production of singlet oxygen is a relatively new field. Through two-photon excitation of a sensitizer, we can study issues and exploit phenomena not possible or relevant with the one-photon counterpart.

Much of the information gleaned from experiments that examine the two-photon sensitized production of singlet oxygen contribute to a better understanding of the fundamental principles of photophysics (*e.g.*, the interaction between the radiation field and a molecule, effect of molecular structure on light-induced transitions between electronic states, *etc.*). However, the two-photon process will probably have its greatest effect, at least from the perspective of someone interested in processes that depend on singlet oxygen, as a tool that allows for unique spatial and spectral control in singlet oxygen production. As such, it will facilitate mechanistic insight, particularly in complicated heterogeneous systems (*e.g.*, a mammalian cell). Of course, one technique is not a panacea. This is certainly true in the present case. Nevertheless, it will nicely complement many experiments that use protein-confined and -localized sensitizers, for example.

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Chapter 8

Activatable Photosensitizers

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8.1. Introduction

Conventional singlet oxygen ($^1\text{O}_2$) photogeneration is a tristimulus process that requires the simultaneous combination of a photosensitizer (PS), light, and oxygen. Activatable photosensitizers (aPSs) are a special type of PSs whose activity can be turned on by a wide variety of molecular stimuli. This allows a more precise control on $^1\text{O}_2$ generation, thereby improving the selectivity and safety during photosensitization processes.¹⁻³ Their general principle of action is that an aPS is maintained in a quenched state until a molecular activation step takes place that relieves its ability to photosensitize $^1\text{O}_2$ (Figure 8.1). As such, aPSs can be considered to be examples of 3rd generation PSs.⁴ This chapter reviews the known aPSs, the reported mechanisms of activation, and the main stimuli used to control singlet oxygen generation.

A large portion of aPS can be used in theranostics. This is due to the fact that, usually, an increase in $^1\text{O}_2$ production is coupled with a variation in fluorescence.^{2,5} They can also be used as activatable fluorescent imaging probes because they share similar activatable mechanisms as for $^1\text{O}_2$ generation.⁶ Even if the fluorescence increase is not too impressive, they can be useful to image the localization of the PS and to determine the degree of PS uptake.⁷ This is very useful from a medical perspective. For example, in photodynamic therapy (PDT), aPS are used as aids in defining and adjusting therapy parameters. A number of molecular approaches have emerged over the last few years, as described below.

8.2. Activation Mechanisms

Effective photosensitization of $^1\text{O}_2$ requires absorption of light by the PS, formation of a long-lived excited state, energy transfer to molecular oxygen, and release of the caged $^1\text{O}_2$ from the PS vicinity to the external bulk media.⁸ Each of these steps is amenable to quenching, therefore rendering the aPS inactive (Figure 8.2). The following sections describe specific approaches of activation mechanisms in detail.

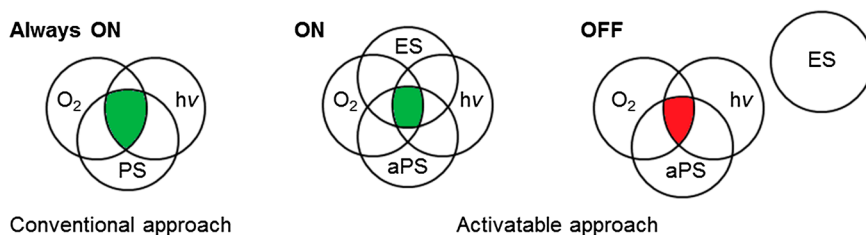


Figure 8.1. Venn diagram with the requirements for $^1\text{O}_2$ generation. From a conventional approach, the encounter must be between O_2 , light and a PS. From an activatable approach, selectivity is increased by a fourth factor: an external stimulus (ES). $^1\text{O}_2$ production (green intersection) is achieved only when all four factors get together.

8.2.1. Self-Quenching

Self-quenching phenomena (SQ) refers to the scavenging of aPSs* by interaction with a ground-state PS. In most cases, this requires close vicinity between PS molecules, and therefore, the relief mechanism is due to an increase of the distance between these (Figure 8.3(A)). The SQ concept has been elegantly pushed to the limit in systems such as porphyrin nanodiscs⁹ and porphysomes.¹⁰ Porphysomes are self-assembled porphyrin aggregates with 99% quenched fluorescence. Interaction of porphysomes with cells leads to a structure disruption, causing a $^1\text{O}_2$ quantum yield increase up to 12-fold.¹¹

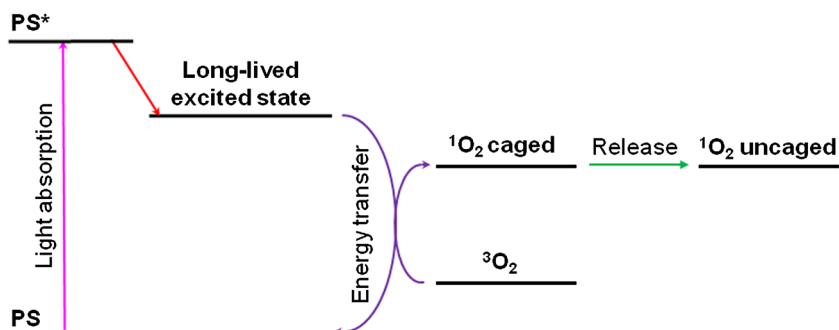


Figure 8.2. Jablonski diagram presenting possible photophysical/photochemical events that can be quenched. Magenta color corresponds to the absorption of light, red color presents the formation of a long-lived excited state (singlet-triplet states), violet color is the energy transfer process from $^3\text{PS}^*$ to O_2 and green color the possible competition between deactivation and release of $^1\text{O}_2$ to the external bulk media.

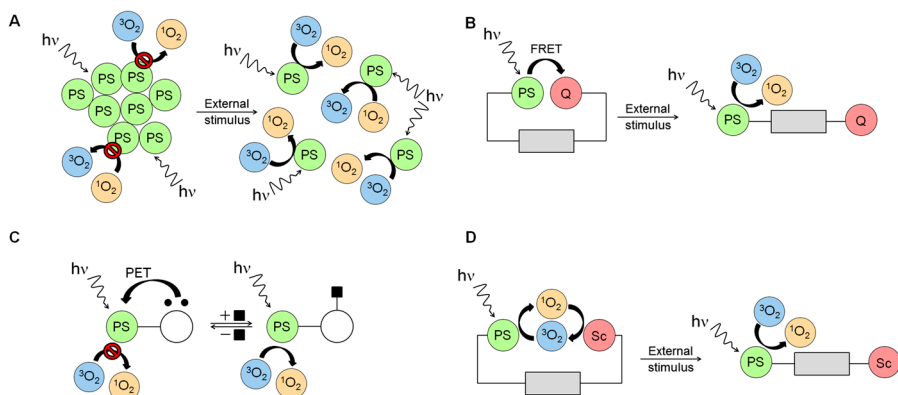


Figure 8.3. The different activation mechanisms presented. (A): self-quenching (SQ). (B): Förster resonance energy transfer (FRET). (C): photoinduced electron transfer (PET). (D): $^1\text{O}_2$ scavenging.

8.2.2. Energy Transfer

Förster resonance energy-transfer (FRET) phenomena can be applied to aPSs. The PS and a suitable energy-transfer acceptor (Q) are held together in an initial conformation, thereby enabling efficient deactivation of the PS* through nonradiative dipole–dipole coupling.¹² Since FRET efficiency is proportional to the reciprocal sixth power of the distance between donor and acceptor, an external stimulus that changes the donor–acceptor distance will have a large impact on FRET efficiency. Therefore, the PSs* ability to interact with oxygen will vary (Figure 8.3(B)). Typically, acceptors that dissipate energy as heat from PSs* are preferred to other radiative mechanisms. These are the so-called “dark quenchers”.¹³

8.2.3. Electron Transfer

Photoinduced electron transfer (PET) is an excited state electron-transfer process by which an excited electron is transferred from donor to acceptor. Due to PET, a charge separation is generated. This electron transfer can effectively quench the PS*, thereby shutting down $^1\text{O}_2$ production.^{14,15} In contrast, $^1\text{O}_2$ generation would occur if this electron transfer were blocked by external stimuli (Figure 8.3(C)). Usually, this electron transfer occurs from a nonbonding electron lone pair, like an amine (usually tertiary amines) or ethers.

8.2.4. $^1\text{O}_2$ Scavenging

The previous strategies try to avoid $^1\text{O}_2$ generation. However, $^1\text{O}_2$ scavenging strategy attempts to react specifically with $^1\text{O}_2$ before it exits the system. When the system is activated by external stimuli, PS and scavenger are separated, reducing $^1\text{O}_2$ scavenging effectivity (Figure 8.3(D)). Carotenoids are typically used as scavengers. They are well known as antioxidants in animals and as photoprotective agents in the photosynthetic system of plants. For example, $^1\text{O}_2$ is diffusionally quenched by β -carotene ($k_{\text{quench}} = 1\text{--}3 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$).^{16,17}

8.3. External Stimuli

The activation of an aPS must be carried out by a specific external stimulus. These stimuli can be either from a molecular recognition, by means of coupling of small molecules, such as protons, or by media properties, like oxidative stress or viscosity. This can be visualized as a molecular “Trojan horse” system. These types of molecules can quickly change into an aggressive form, able to kill cells (both tumorous and microbial).

8.3.1. Molecular Recognition Activation

The term *molecular recognition* refers to the specific interaction between two or more molecules/moieties through noncovalent bonding.¹⁸ A typical example is the use of base pairing in DNA to construct molecular beacons. Molecular beacons are hairpin-loop structures that hold a fluorophore and a quencher together, which results in PS* quenching. When the beacon encounters a specific DNA sequence, the loop portion of the strand changes its conformation to bind with the target sequence (external stimulus) and thereby causing the separation of the two moieties and restoring fluorescence.^{19,20}

This concept has been translated to PDT since “photodynamic molecular beacons” (PMB) have been developed. They enable the control of the PS ability to produce $^1\text{O}_2$ through DNA/RNA recognition (nucleic acid-based photodynamic molecular beacons or NAPMBs).²¹ In NAPMBs, one end of a single DNA strand is labeled with a PS, whilst the other end has a quencher or another PS moiety allowing SQ.²² When the strand folds, the two moieties come close together, preventing $^1\text{O}_2$ production (Figure 8.4(A)).^{23,24}

A similar approach would be to label two separated DNA strands with a PS and a quencher, respectively.²⁵ When the beacon comes in contact with the target DNA sequence, the quencher breaks apart and $^1\text{O}_2$ production is re-enabled (Figure 8.4(B)). The nucleic acid target can either displace partially or completely the original complementary DNA strand.

aPS by molecular recognition can be expanded to other biomolecules thanks to aptamer technology. An aptamer aPS is mainly composed by three

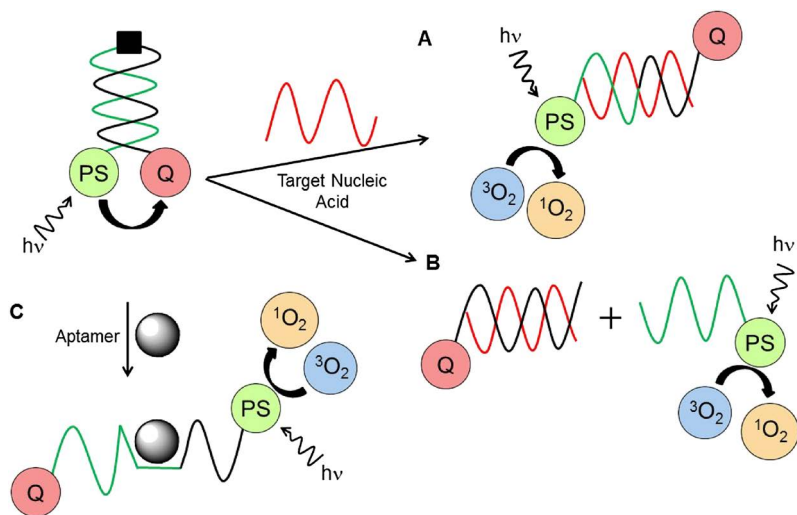


Figure 8.4. (A) and (B) present a schematic functional description of nucleic acid-based photodynamic molecular beacons. Originally the PS and quencher, either on the same (A) or on different DNA strands (B), are held close together. In the presence of the target DNA sequence, the two moieties separate and $^1\text{O}_2$ production is restored. (C) presents the schematic functional description of an aptamer aPS.

components: an aptamer, a partial complementary DNA strand, and a polyethylene glycol linker uniting these two moieties (Figure 8.4(C)). Aptamers are single-stranded DNA or RNA chains that are able to recognize and bind to target biomolecules with high affinity and specificity.^{26,27} The PS and Q are covalently attached to the two ends of the chain. This selectivity has been proved upon ATP and α -thrombin aptamers, which are specific to these analytes in front of other nucleotides (UTP, GTP or CTP) and proteins (BSA, IgG or IgM), respectively.²⁸ Furthermore, aptamers can recognize targeted cells.²⁹ Also, aptamer aPS have been conjugated with gold nanoparticles³⁰ and single-walled carbon nanotubes (SWNT)³¹ for their use as singlet-state quenchers. Initially, the PS is near to a gold nanoparticle/SWNT, and therefore, $^1\text{O}_2$ is quenched by energy transfer. When the aptamer is introduced into the system, the distance increases allowing $^1\text{O}_2$ generation. It is important to remember that some PS can either increase or decrease $^1\text{O}_2$ generation simply upon direct binding to nucleic acids.^{32,33}

8.3.2. Enzyme Activation

In addition to the DNA/RNA chains previously exposed, peptides can also be used to construct molecular beacons, termed *peptide-based photodynamic molecular beacons* (PPMBs). PS and quencher are initially held close together by the peptide conformation. The separation of both molecules takes place due to specific activity of proteases that cleave the linking sequence (Figure 8.5).^{20,34–36} The most used peptide linkers are those where the substrates are selective to caspase-3 protease,^{35,37} matrix metalloproteinases^{38,39} and fibroblast activation protein.⁴⁰ Caspase-3 protease is one of the proteins responsible for cell-apoptosis, whilst matrix metalloproteinases are overexpressed proteins in vertebral/bone metastases, especially in breast cancer. An expected advantage of this approach was that phototoxicity would be strongly amplified by enzymatic turnover.

A known problem of PPMBs is the dependence of quenching on the folding of the linker peptide that is recognized by the enzyme.^{20,41} The elegant concept of “zipper molecular beacons” has been devised to overcome this drawback, in which a pair of polycation and polyanion arms hold the PS and quencher in close proximity by electrostatic interaction. When the stimulus takes place, an enzymatic cleavage dissociates the quencher and restores the PSs activity.^{41,42} In addition to FRET quenchers, molecular beacons that use $^1\text{O}_2$ scavengers have also been constructed.⁴³ In both types of molecular beacons there is typically a *ca.* 10-fold factor between the *ON/OFF* states.

Another option is to use polypeptides. For example, coupling multiple PS molecules onto a polylysine peptide induces PSs aggregation and self-quenching. $^1\text{O}_2$ generation is expected to increase when the peptide linkages of the polylysine backbone are cleaved by tumor-associated enzymes.^{44,45} This degradation is expected to occur mainly by lysosomal cysteine and serine-proteases. A possible improvement would be to introduce an enzyme target between the polymeric backbone and the PS. An enzyme would cleave the target, and

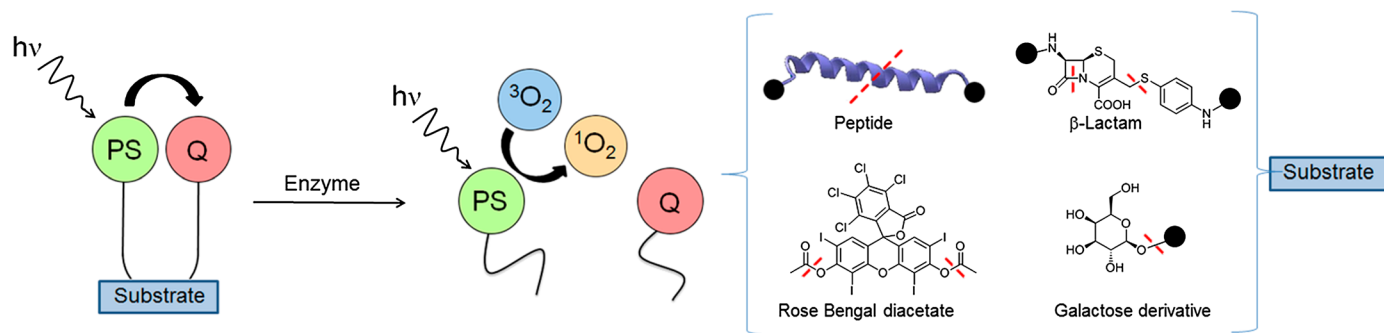


Figure 8.5. Schematic functional description of an enzymatic aPS. The linkers used are a polypeptidic chain for proteases, β -lactam (cephalosporin) for β -lactamase, ester bonds for esterases and galactose conjugates for β -galactosidases.

therefore, selectively activate $^1\text{O}_2$ production.⁴⁶ This could be accomplished by the use of specific secondary cleavable peptidic linkers tethering the PS to the polymer backbone.^{47,48} Examples of specificity are UPA (a protease overexpressed in prostate cancer)⁴⁹ or thrombin (protease upregulated in synovial tissues of rheumatoid arthritis).^{50,51} This last type of cleavage does not need the backbone to be a peptide, it can be a polymeric chain⁵² or a gold nanorod.⁵³ In this last case, the gold nanorod acts also as Q by energy transfer.

Enzyme activation has also been used to release a PS from a photoinactive prodrug. For instance, rose bengal (RB) can be released from its diacetate derivative by the action of cellular carboxylic esterases.^{54,55} On the one hand, the acetate moieties quench the fluorescence and photosensitization properties of RB because they are able to affect the π electron system of the chromophore. On the other hand, they facilitate cell internalization and accumulation, due to an increase in the hydrophobicity character of the cellular media (RB in biological-pH has an anionic structure).⁵⁶ Uncaged RB is redistributed through the cytoplasm, allowing $^1\text{O}_2$ generation and consequently inducing multiple organelle photodamage,⁵⁷ especially in Golgi apparatus,⁵⁸ mitochondria and endoplasmic reticulum.^{55,59} The same strategy has been applied to other aPSs such as Hypocrellin B-acetate, which can produce $^1\text{O}_2$ but also other ROS species by a type-I mechanism.^{60,61}

Another enzyme that has been used to release a PS from a prodrug is β -galactosidase. It has been recently used to release thiazole orange derivatives from their galactose conjugates.⁶² Like acetate in the previous case, $^1\text{O}_2$ generation is quenched by the galactose moiety and facilitates cell internalization. Recently, other PSs have been used like Tokyo-Green iodated derivatives⁶³ or selenium-substituted xanthenium dyes⁶⁴ with a fold activation of 20 and 300, respectively. It is possible to achieve this high activation fold thanks to a shift in absorbance spectra when irradiating with a monochromatic laser (532 nm). It has been proved in cells that its phototoxicity is extremely dependent on β -galactosidase concentration.⁶⁴

A last example is β -lactamase enzyme activation. This type of aPS is important due the abuse of antibiotics by our society. Unfortunately, this abuse has caused a heavy increase in the number of bacteria with β -lactamase enzymes.⁶⁵ The chemical structure of β -lactam aPS consists of a cephalosporin core (a class of β -lactam antibiotic) covalently bonded with two chromophoric moieties on either side of the molecule.⁶⁶ If the two chromophoric moieties are PSs, $^1\text{O}_2$ generation is mainly self-quenched.⁶⁷ Also, it is possible to reduce even further $^1\text{O}_2$ generation in the OFF state by using a dark quencher (BHQ-3) instead of a self-quenching mechanism.⁶⁸ This approach allows use of inorganic ruthenium complexes as theranostic agents, being both fluorophore and PS at the same time.⁶⁹

The activation by β -lactamase takes advantage of bacterial resistance mechanisms. β -lactamase cleaves the β -lactam ring, releasing the PS and allowing $^1\text{O}_2$ generation.⁶⁷ The main advantage in front of nonphotochemical

treatments is that PDT treatment should not develop bacterial resistance, giving us another tool to kill resistant bacteria.^{70–72}

8.3.3. pH Activation

Other external stimulus that can be used is pH. pH-aPS have attracted considerable interest because the acidic microenvironment of a tumor is different from healthy tissues (pH 6.5–6.8 vs. 7.4).⁷³ In addition, the significantly increased acidity in subcellular compartments of cancer cells such as lysosomes (pH 4.5–5.0)^{74,75} may promote even further the generation and release of $^1\text{O}_2$. For decades, the pH-dependent behavior of some PS, such as porphyrins, chlorins, chalcogen-pyrylium dyes, or phenylene vinylenes have been studied, but in this chapter we will focus on the newest strategies on pH activation.^{76–79}

PET has been widely exploited as a pH-activatable mechanism, due to the acid–base equilibrium that is able to activate/deactivate free electronic pairs.^{80,81} The first example used as an aPS is a two-component system formed by an azadipyrromethene covalently bound to a tertiary amine.⁸² In basic media, the non-bonding electron pair of the amine's nitrogen atom is available for PS quenching by PET (Figure 8.6(A)). In acidic media, the amine is protonated and can no longer quench the PS. $^1\text{O}_2$ production in acidic media is around 9-fold higher compared to basic media. It is possible to tune the switching conditions changing the pK_a of the amine by electronic or steric effects.^{83,84} Since then, other aPSs have been developed following the same design principle. Some examples are: (i) selenium–rubiyrin,⁸⁵ phthalocyanines⁸⁶ or azaphthalocyanines⁸⁶ with pendant amino moieties; (ii) imidazole-modified porphyrins, in which imidazole is

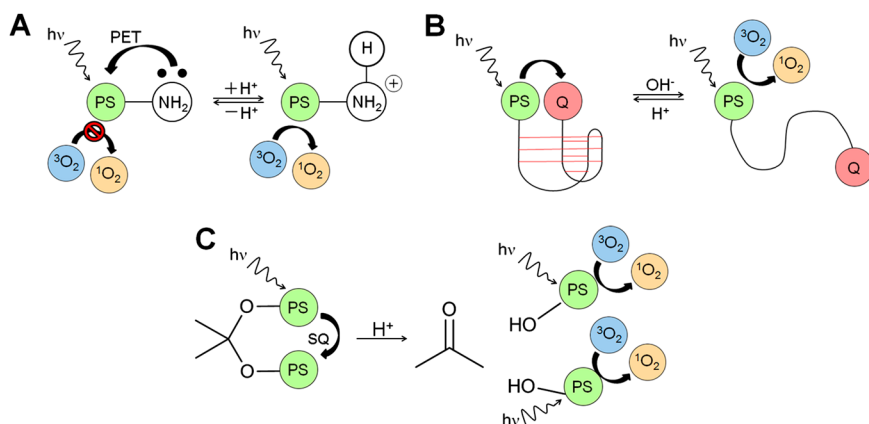


Figure 8.6. Schematic functional description of pH aPS. (A): acid–base equilibrium where a free electronic pair can be protonated and disfavors PET phenomena. (B) Conformational change of DNA i-motifs induced by basic medium, in which some residues are deprotonated. (C) Ketall evolution to ketone due to an acidic environment, causing the PS release.

the acid–base moiety,⁸⁷ or (iii) axially substituted tetra-aminosilicon(IV) phthalocyanine (metal complex) where in the axial substitution there are one⁸⁸ or more amine^{89,90} moieties capable of being protonated or deprotonated.

In addition, a variety of macromolecular pH-sensitive moieties have been developed to link the PS and the Q.^{91,92} For instance, the conformation of DNA i-motifs^{93,94} keeps the Q close to the PS in acidic media, preventing $^1\text{O}_2$ generation. On the other hand, in basic media the deprotonation of some residues induces a conformational change separating the two moieties and allowing efficient $^1\text{O}_2$ generation (Figure 8.6(B)).⁹¹

Another option is a polysaccharide/PS conjugate that consists of a glycol chitosan backbone multiply bound to DEAP (3-diethylaminopropyl isothiocyanate, used as pH-sensitive moiety), chlorin e6 (used as PS), and polyethylene glycol.⁹² In basic media, the aPS is SQ, but in acidic media, the polysaccharide/PS conjugate will undergo conformational changes into an uncoiled structure allowing $^1\text{O}_2$ generation. The pK_b of this system is close to 6.8.⁹⁵

Finally, other approaches have been developed taking advantage of an acid-cleavable ketal moiety.⁹⁶ This aPS is a phthalocyanine dimer linked by a ketal moiety. Due to the propensity of phthalocyanines to form H-type dimers, allowing quenching of $^1\text{O}_2$ production. However, under acidic conditions ($\text{pH} < 6.5$), the ketal linker is cleaved and the two phthalocyanine units are separated, restoring its $^1\text{O}_2$ generation (Figure 8.6(C)).⁹⁷

8.3.4. Small-Molecule Activation

In Sections 8.3.1 and 8.3.2 the activation is achieved by big molecular assemblies and in Section 8.3.3 by pH, which can be considered the first example of small-molecule activation, because the activation is triggered by H^+ . Another example is the crown-ether-based PET modulators because they are selective to some cations. In the absence of that cation, $^1\text{O}_2$ generation is quenched by PET. But when a cation is introduced into the moiety, the lone electronic pair interacts preferentially with the cation, allowing $^1\text{O}_2$ photosensitization (Figure 8.7(A)).⁹⁸ It has also been used in aptamer recognition for small biomolecules, like ATP.²⁸

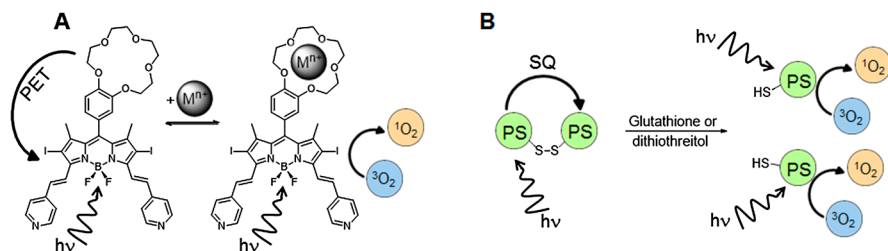


Figure 8.7. Schematic functional description of small-molecule aPS. (A): crown-ether activation *via* PET mechanism. (B): PS activation *via* reduction of the disulfide bridge by glutathione or dithiothreitol.

Another targeting functional group is the thiol moiety, such as glutathione or dithiothreitol, because they are markers of reducing environments.⁹⁹ These types of aPSs have the PS and the Q separated by a linker that contains a disulfide bond. When the bridge is reduced (by glutathione^{100,101} or dithiothreitol¹⁰²), the static quenching is reduced and $^1\text{O}_2$ is generated (Figure 8.7(B)). Also, glutathione has been used to break sulfonate esters, so that the quencher can leave the system permitting $^1\text{O}_2$ production, and therefore, can be used as an activation strategy.¹⁰³ Further developments in this field have been accomplished. Examples are PSs covalently bound to disulfide bridges linked to a polymer (hyaluronic acid backbone)¹⁰⁰ and graphene oxide nanoparticles.¹⁰¹

8.3.5. Light Activation

Light is an obvious external stimulus and, not surprisingly, a number of studies have focused on coupling a PS to a photochromic switch.¹⁰⁴ Ideally, the *ON/OFF* states of the photoswitch have very different quenching abilities. An elegant example has been recently disclosed,¹⁰⁵ whereby a photoswitchable derivative diarylethene has been combined (noncovalently) with zinc-tetraphenylporphyrin (ZnTPP) (Figure 8.8(A)). The relative energy of the triplet excited states of the porphyrin and of the two forms of the diarylethene are 1.23 eV for the closed isomer, 1.61 eV for ZnTPP, and 2.89 eV for the opened form. This structural change results in a 93% decrease in the $^1\text{O}_2$ production quantum yield when the switch is in the closed form, due to the shutdown of energy transfer.

A possible improvement is the use of a binary combination of metalorganic frameworks (MOFs). One of them is a photochromic porous coordination network with a diarylethene as photochromic moiety, while the other is a singlet oxygen-generating porous coordination framework with tetrakis(4-carboxyphenyl)-porphyrin (TCPP) as a PS.¹⁰⁶ Another option is to take advantage of a merocyanine-type photoswitchable acid. When irradiated with UV light, a release of protons to the media takes place. Then, by means of pH-dependent PS, such as an osmium polypyridine complex, $^1\text{O}_2$ is generated (Figure 8.8(B)).¹⁰⁷

8.3.6. Viscosity Activation

Viscosity is a macroscopic property that can be used as an internal stimulus. It has been demonstrated that the viscosity of intracellular domains can vary from 1 cP (pure water) to 600 cP (inside a cellular membrane).^{108,109} This type of aPS is zinc-porphyrin dimers. The linker is a 1,3-butadiyne group, the rotation of which is partially hindered in high viscosity.¹¹⁰ This allows the formation of two different singlet states (planar and twisted conformers) in high-viscosity media. However, in low-viscosity media, the twisted conformer evolves to the planar one. The intersystem crossing efficiency

(and consequently $^1\text{O}_2$ generation) is much lower for the twisted singlet state than for the planar state. Also, the excitation wavelength is different for both species, so it is possible to excite preferentially one over the other.¹¹¹ Taking advantage of these properties, a viscosity- and wavelength-dependent PS can be obtained.

8.3.7. Multiple Stimuli Activation

In the previous sections, activation happens only by means of one external stimulus, but it is possible to achieve multiple activation steps. They are examples of an “AND” chemical logic gate with two input channels, which should be a possible chemical alternative to the silicon-based digital electronics.¹¹² This system would release $^1\text{O}_2$ when two related cellular parameters are above a threshold value within the same spatiotemporal coordinates (Figure 8.9).^{98,113} One approach is *via* crown-ether-based PET modulators, which are sensitive to certain cations, like sodium. If the PS is also linked to a pH-sensitive moiety, the concentration of H^+ will also be a quenching parameter. When both stimuli are present, $^1\text{O}_2$ production is relieved.⁹⁸ This dual approach was further developed to obtain PET quenchers that are active in hydrophobic environments, and thereby, preventing the production of $^1\text{O}_2$ when the PS is localized in hydrophobic pockets of cellular proteins.¹¹⁴

Another approach is an unsymmetrical silicon(IV) phthalocyanine, in which the two axial ligands have a cleavable moiety (disulfide or hydrazine) with a Q (ferrocenyl) at their extremes. They can be cleaved by dithiothreitol (as an example of reducing agent) and acid media, respectively, allowing $^1\text{O}_2$ generation when the two moieties are broken.¹¹³

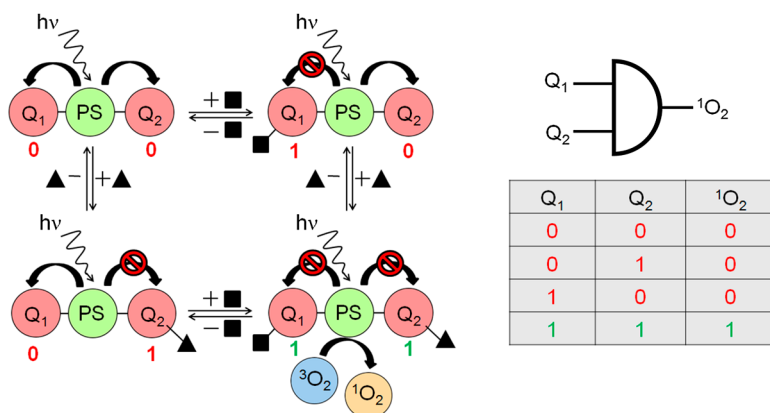


Figure 8.9. Schematic functional description of multiple stimuli aPS, in which all moiety quenchers are required to be in OFF state to generate $^1\text{O}_2$ photosensibilization.

8.4. Summary and Outlook

aPSs introduce a fourth control factor in $^1\text{O}_2$ -generation selectivity. This new control factor is achieved by an external stimulus that triggers $^1\text{O}_2$ production.

The external stimuli can be done by different aPS activation pathways. Known quenching phenomena such as self-quenching, energy transfer, electron transfer or even scavenging, impede the operative formation of singlet oxygen. By addition of the external stimuli, $^1\text{O}_2$ is formed in small spatiotemporal coordinates.

These external stimuli may consist in a slight change in pH (subtle difference between a tumorous and healthy cell), the presence of an enzyme or a nucleic acid chain and even a cation. To sum up, a change in the system must be induced to activate the singlet oxygen generation process. This new type of PS broadens the variety of singlet oxygen producers and should be used in the near future as highly specific targeting systems to face diseases from microbial, viral or carcinogen origin.

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Chapter 9

Heterogeneous Singlet Oxygen Sensitizers

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9.1. Introduction

The involvement of singlet molecular oxygen in photosensitized oxidations was postulated in experiments carried out in heterogeneous systems. Kautsky observed in the 1930s that absorption of light by suitable dyes adsorbed on the surface of silica gel or aluminum oxide gel resulted, in the presence of minute quantities of molecular oxygen, in the oxidation of molecules adsorbed on different particles of the same supporting material.^{1,2} The occurrence of phosphorescence quenching by oxygen, both in the presence and in the absence of the oxidizable molecules, was interpreted as a sign of energy transfer to oxygen, with the formation of one of its lowest excited singlet states. Singlet molecular oxygen had to diffuse during its lifetime from the vicinity of the sensitizer to the place where the acceptor was located. For diffusion to take place, total pressures had to be enough low. However, Kautsky's explanation was immediately rejected^{3,4} and, according to Kearns, it remained unrecognized until his death in 1966.⁵ The accepted explanation was the involvement of a "sensitizer oxygen complex".⁶ It was mainly the work of Foote that confirmed the participation of the lowest excited singlet state, $^1\text{O}_2$ ($^1\Delta_g$), in sensitized photooxidations.^{7,8} In what follows, $^1\text{O}_2$ will refer specifically to this state.

Reactants bound to insoluble supports like organic polymers, ion exchange resins, silica, alumina, glass beads, *etc.* have been used since long in organic synthesis⁹ and the interest in the field was renewed in terms of the concept of Green Chemistry;¹⁰ $^1\text{O}_2$ -driven photooxidation constitutes a relevant chapter in this context.¹¹ After the work of Foote and others during the 1960s, the field of heterogeneous photosensitization evolved quickly and many dyes – mainly visible light-absorbing xanthenes, porphyrins, phthalocyanines and ruthenium complexes – and supporting materials – both organic and inorganic – have been the object of intense research during the last forty years. Adsorption, embedding, electrostatic binding, complexation, and covalent linkage were used as the main techniques to join dyes and supporting materials. Aside from concentration quenching resulting from the presence of dyes at high local concentrations and, in most cases, the occurrence of light scattering – effects that will be addressed in Section 9.3 – insoluble bead-like and film photosensitizers have as advantages, among others, the easy separation and recuperation of the material and the possibility of its reuse, as far as dye leakage or chemical attack are negligible. Mostly particulate systems will be considered in what follows. Relevant aspects in the field have been addressed comprehensively in the literature from different points of view, *e.g.* chromophores in porous silicas and minerals,¹² polymeric supports,¹³ synthetically useful singlet oxygen,¹⁴ singlet oxygen in zeolites,¹⁵ immobilized photosensitizers in general,^{16,17} singlet oxygen in polymers,¹⁸ and new materials for photooxygenation.¹⁹ Therefore, a limited series of case examples will be given in Section 9.2, where different ways of obtaining heterogeneous photosensitizers will be presented, with emphasis in the methodology used to quantify their efficiency for the generation of $^1\text{O}_2$. Homogeneous and

microheterogeneous systems will not be considered but, as needed, some examples on soluble and solid homogeneous polymeric photosensitizers will be presented. Generation of $^1\text{O}_2$ using nanomaterials will be covered elsewhere in this volume and, therefore, this kind of material will also not be considered. The way to quantify relevant photophysical parameters for light-scattering systems will be addressed in Sections 9.3 and 9.4, where factors influencing the availability of excited states will be discussed. Finally, general conclusions will be summarized in Section 9.5.

9.2. Survey of Supporting Materials, Dyes and Immobilization Techniques

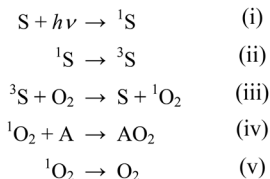
In spite of the reactivity and selectivity of $^1\text{O}_2$, the ease of its production using visible light-activated photosensitizers and the lack of expensive reactants when common triplet state yielding dyes are used, $^1\text{O}_2$ photooxidations driven in homogeneous solution present some drawbacks; as pointed out by Griesbeck *et al.*:²⁰ the photosensitizer and the reactants must be soluble in the same solvent; removal of the dye is often laborious; $^1\text{O}_2$ lifetimes are larger in environmentally unfriendly halogenated solvents; dye photobleaching is often observed; and oxygen purging may be problematic. These and other reasons motivated the search for heterogeneous materials including suitable dyes. Though earlier examples may be found in the literature, in 1973, Schaap, Neckers and coworkers reported on what they qualified as “the first example of a synthetically applicable, polymer-based photosensitizer.” It was synthesized attaching rose bengal to beads consisting of a chloromethylated polystyrene-divinylbenzene copolymer by means of the dye carboxylate, applying a synthetic procedure originally developed by Merrifield.²¹ Using this insoluble photosensitizer, which will be thereafter called P-rose bengal, they performed photooxidation experiments, obtaining product distributions consistent with the involvement of $^1\text{O}_2$. Furthermore, reactions were inhibited by 1,4-diazabicyclo[2.2.2]octane (DABCO) and were insensitive to a typical free-radical inhibitor like 2,6-di-*tert*-butylcresol.

In a subsequent paper, Schaap, Neckers and coworkers performed further studies on P-rose bengal and other photosensitizing dyes bound to the same polymer. They reported for P-rose bengal (200–400 mesh, equivalent to 75–37 μm ; *ca.* 200 μmol rose bengal per g of polymer; dark red beads) in CH_2Cl_2 suspension a $^1\text{O}_2$ -generation quantum yield $\Phi_{\Delta} = 0.43$, which should be compared with $\Phi_{\Delta} = 0.76$ for rose bengal in dilute methanol solution. Quantum yield was measured following the consumption of 2,3-diphenyl-*p*-dioxene under oxygen saturation. Attachment of dyes like eosin Y, fluorescein, chlorophyllin, and hematoporphyrin yielded lower Φ_{Δ} values.²² Ten years later, Neckers reported Φ_{Δ} values as a function of dye loading, from *ca.* 10 to 300 μmol rose bengal per g of polymer, obtaining fluctuating values between 0.76 and 0.91. The author explained the difference assuming that earlier results were obtained at lower oxygen concentrations in the beads. He quotes that P-rose

bengal is compatible with nonpolar solvents, stable against light and oxygen and does not undergo energy-wasting processes (see Section 9.3) because dye molecules are essentially isolated from one another by the polymer backbone.^{23,24} It is surprising that Φ_{Δ} values exceed those of rose bengal commonly found in solution. Values of Φ_{Δ} measured by continuous photolysis for rose bengal and rose bengal alquilammonium salts attached to chloromethylated poly(styrene-*co*-divinylbenzene) and poly(styrene-*co*-divinylbenzyl chloride) in CH_2Cl_2 are listed in ref. 25. P-rose bengal was commercialized in the USA by Hydron Laboratories, Inc., Brunswick, New Jersey as Sensitox (CAS 94035-42-4) since 1976. The trademark was registered in 1978 and canceled in 1985. Sensitox was patented in Canada²⁶ and the USA.²⁷ Finally, P-rose bengal disappeared from the market in the second half of the 1980s.

P-rose bengal is not wetted by and does not swell in water or alcohol. For that reason, a few years after the synthesis of Sensitox, Schaap *et al.* developed a heterogeneous photosensitizer suited for aqueous and alcoholic systems. It consisted of rose bengal chemically attached to a copolymer of chloromethylstyrene and the monomethacrylate ester of ethylene glycol crosslinked with the bismethacrylate ester of ethylene glycol (37–75 μm beads). A value of $\Phi_{\Delta} = 0.48$ was determined in methanol for this photosensitizer, known as Sensitox II (CAS 94035-43-5).²⁸ In spite of the high quantum yields reported for these heterogeneous photosensitizers, experiments performed by Midden and Wang showed that rose bengal chemically linked to glass beads or adsorbed on glass beads and silica gel and methylene blue adsorbed on glass beads and acidic or neutral alumina, depending on their pore size, were more efficient than Sensitox and much more than Sensitox II. These experiments were performed in a three-phase system – photosensitizer plate/gaseous phase/solution containing the acceptor – thus excluding physical contact between sensitizer and acceptor; the photosensitizer was dry in this case.²⁹ Though experiments do not allow calculation of quantum yields, they are useful in order to compare the rates at which $^1\text{O}_2$ leaves the photosensitizer plates insofar all other variables are kept constant. Results are affected by the diffusion of ground state and excited molecular oxygen molecules and by the lifetime of $^1\text{O}_2$ within the photosensitizer, which are indeed very different from those in suspension. Physical interactions of dyes in the dry, collapsed solid are very different from those found in swelling solvents.

Photooxidation efficiencies obtained by continuous photolysis of photosensitizer suspensions following the consumption of a well-known $^1\text{O}_2$ chemical scavenger are affected by oxygen diffusion (arrival of ground state O_2 and exit of $^1\text{O}_2$) and adsorption and by scavenger availability (depending on polymer swelling, physical interactions with the polymer backbone, and adsorption). It must be pointed out that the simplified kinetic formalism used by the authors in ref. 22 and subsequent papers (Scheme 9.1) cannot be handled as a homogeneous mechanism for particulate suspensions and even for polymer solutions, unless all triplet state molecules are quenched by oxygen and all singlet oxygen molecules are scavenged by the acceptor because reactant concentrations are not constant throughout the system.



Scheme 9.1. S: photosensitizer; A: acceptor.

Furthermore, it is risky to extrapolate kinetic constants obtained in homogeneous systems to the heterogeneous systems into consideration as they are generally affected by the microenvironment. On the other hand, calculation of absorptances[†] in suspension is a very difficult task: even if light transmission can be ruled out, as demonstrated by Schaap and Neckers, backscattering may reduce the fraction of incoming light absorbed by the sample. Finally, concentration quenching and dye aggregation may take place. However, almost all these factors will result in the underestimation of Φ_{Δ} . The very high rates of scavenger consumption obtained for P-rose bengal, leading to unusually high Φ_{Δ} values, might be the result of the occurrence of chemical reactions different from ${}^1\text{O}_2$ attack or simply an expression of the large errors commonly found in the quantification of heterogeneous systems.

Absorption spectroscopy generally yields relevant information about the environment in which the dye is located and, in particular, whether the dye is in its monomeric or in an aggregated state. When dealing with light-scattering systems resource has to be made to reflectance spectroscopy. Usually, diffuse reflectances, R , of optically thick[‡] samples are converted into Kubelka–Munk, called also remission, function values through:³⁰

$$F(R) = \frac{(1 - R)^2}{2R}. \quad (9.1)$$

For dilute, isotropically scattering samples, assuming that the supporting material does not absorb light at the wavelengths of interest,

$$F(R) = k/s = 2\varepsilon C/s, \quad (9.2)$$

where k and s are the absorption and scattering factors of the sample, and ε and C are the Napierian molar absorption coefficient and the molar concentration of the dye, respectively. At high loadings, the reflectance may be so low that the sample has to be diluted with a particulate solid with low absorption, usually a reflectance standard.³¹ In this case, as far as mixing

[†]Absorptance is the fraction of incoming radiation absorbed by the sample at a particular wavelength.

[‡]A light-scattering medium is optically thick when transmitted radiation is negligible.

does not modify the scattering coefficient of the diluent solid, eqn (9.1) can be replaced by:

$$F(R) = \frac{(1-R)^2}{2R} \frac{W}{W^*}, \quad (9.3)$$

where W^* is the weight of the sample mixed with the diluent and W is the weight of pure sample that would be needed to fill the whole volume of the sample holder.

Schaap *et al.* measured the diffuse reflectance spectrum of P-rose bengal diluted with MgO. Results in Figure 9.1 show the remission function spectrum calculated from ref. 22 after digitalization of the published diffuse reflectance spectrum. As W/W^* is not known, $F(R)$ is given in arbitrary units. For comparison, the normalized spectrum of dilute rose bengal in ethanol solution is given. Unfortunately, no further P-rose bengal spectra have been found in the current literature. Paczkowski and Neckers synthesized soluble analogs of P-rose bengal based on a poly(styrene-vinylbenzyl chloride) copolymer and registered their spectra in CH_2Cl_2 as a function of dye loading. Spectra are similar to that for rose bengal in ethanol but shifted *ca.* 15 nm to the red, while the low-energy band is somewhat broader and the high-energy satellite grows in amplitude as dye concentration increases.³² Band broadening and changes in band ratio are typical effects of dye aggregation, whereas band shifts are related to environment changes. Absorption spectra are very different from the remission function spectrum of P-rose bengal, revealing extended dye aggregation in the dry solid. Naturally, in the absence of a swelling solvent, the polymer collapses and interactions among dye molecules increase and lead to the formation of dimers and higher aggregates.

Burguete *et al.* synthesized monolith polymers with high porosity based on *p*-chloromethylstyrene and divinylbenzene adding a porogenic mixture

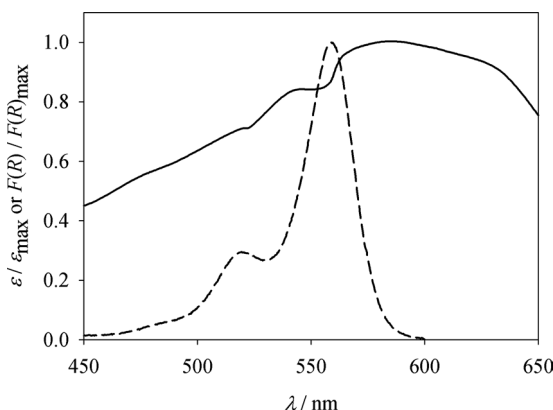


Figure 9.1. Remission function spectrum (full line) of P-rose bengal (calculated from ref. 22) and absorption spectrum (broken line) of rose bengal in basic ethanol (from ref. 33).

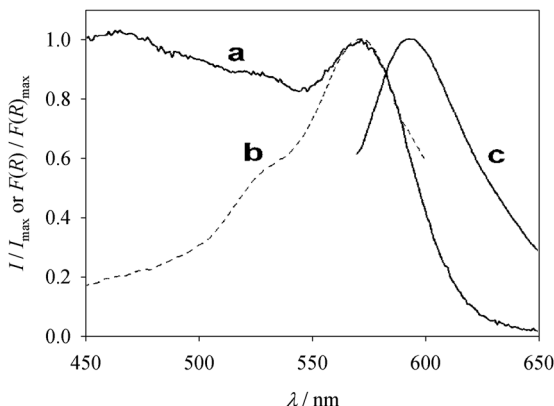


Figure 9.2. Remission function (a), fluorescence excitation (b, $\lambda_{\text{em}} = 625$ nm) and fluorescence emission (c, $\lambda_{\text{ex}} = 550$ nm) spectra of rose bengal modified porous *p*-chloromethylstyrene-divinylbenzene particles. Reproduced from ref. 34 (supporting information) with permission from the European Society for Photobiology, the European Photochemistry Association, and The Royal Society of Chemistry.

of dodecanol and toluene, to which rose bengal was attached. The material was ground obtaining micrometer sized particles, whose spectra are shown in Figure 9.2.³⁴ The excitation spectrum, originated essentially in rose bengal monomers, overlaps reasonably well with the low-energy band of the reflectance spectrum, showing that the dye is essentially monomeric in the dry solid. The analysis of the material by hydrolysis yielded 2 μmol rose bengal per g of resin (far less than for P-rose bengal synthesized by Schaap and Neckers). The authors compared the activity in the oxidation of 9,10-diphenylanthracene in methanol suspension by irradiation at 556 ± 7 nm against P-rose bengal beads synthesized in the same laboratory, containing 160 μmol rose bengal per g of resin (in the order of the most concentrated samples from Schaap and Neckers). In all cases they obtained photooxidation rates up to six times greater for the monolithic material and, strikingly, twice as large as for rose bengal in solution at the same dye volumetric concentration. No data is given for the calculation of the fraction of light absorbed by the dye. The greater activity compared with P-rose bengal is understandable because the monolithic material has probably larger pores but the larger activity than rose bengal in solution is very difficult to understand. It should be noticed that methanol is not the best suited solvent for this kind of materials.

Together with rose bengal, dyes like acridine orange, chlorophyllin, crystal violet, eosin Y, fluorescein, flavin mononucleotide, hematoporphyrin, hemin, malachite green, methylene blue and rhodamine B have been linked to different materials, among them poly(2-hydroxyethyl)methacrylate-ethylene glycol, poly(vinyl formal), bromomethylated borosilicate glass, styrene-maleic anhydride copolymer, cotton, and wool. However, no details on the reactivity of these systems are given.²⁷ Rose bengal, eosin and methylene blue were electrostatically bound to an oppositely charged ion exchange

resin and tested for the photooxidation of 2-methyl-2-butene and anthracene, substrates that undergo characteristic reactions with $^1\text{O}_2$.³⁵ Rose bengal was attached to NovaSyn® TG (chloromethylated styrene/divinylbenzene copolymer linked to polyethyleneglycol chains terminated by amino groups) and used to produce photooxidations in water.³⁶ No Φ_Δ values were reported in the last two cases. Aluminum tricarboximonoamidophthalocyanine (ALTCPc) was bound to Amberlite IRA-93 by linkage with the amine groups of the resin in dimethylformamide.³⁷ The phthalocyanine remains monomeric and is mainly located near the surface of the particles but the high local concentrations attained result in very low Φ_Δ values.

Silica monoliths with embedded or grafted cyanoaromatic dyes were prepared by Lacombe *et al.* for the solvent-free production of $^1\text{O}_2$ at the solid/gas interface and oxidation of dimethylsulfide was investigated. Monoliths were transparent, simplifying greatly the determination of Φ_Δ . For embedded benzo[*b*]triphenylene-9,14-dicarbonitrile, $\Phi_\Delta = 0.89$ was obtained by phosphorescence measurements, assuming $\Phi_\Delta = 1$ for the reference employed, 1*H*-phenalen-1-one. The $^1\text{O}_2$ lifetime within the monolith was low, around 25 μs , most probably due to the presence of residual water and methanol.³⁸ A silicon phthalocyanine was recently bound to aminopropylsilica with increasing chain lengths by condensation of one or more 3-bromopropylamine hydrobromide molecules by Kuznetsova *et al.* with the object of inactivating bacteria through photodynamic action. Though absorption and emission spectra and relative Φ_Δ values, determined in suspensions stabilized by sodium dodecyl sulfate, did not show any difference, the efficiency of photoinactivation of *E. coli* increased with chain length.³⁹ The proximity of the sensitizer to the bacterial membrane surely influences positively the photodynamic activity.

Amore *et al.* joined ALTCPc and rose bengal by chemical linkage and adsorption to various solid supports and studied them in suspension of different solvents. The best results were obtained for rose bengal adsorbed on silica gel ($\Phi_\Delta = 0.25$), in toluene suspension to avoid dye desorption, and ALTCPc bound to silanized silica ($\Phi_\Delta = 0.14$) and to silanized silica gel modified with polylysine ($\Phi_\Delta = 0.18$) in water suspension (for ALTCPc in solution of dimethylsulfoxide $\Phi_\Delta = 0.35$). Almost no $^1\text{O}_2$ was produced by ALTCPc linked to NovaSyn® in ethanol suspension.⁴⁰ As $^1\text{O}_2$ chemical scavenger imidazol was used, following the bleaching of added *N,N*-dimethyl-4-nitrosoaniline.⁴¹ In ethanol and toluene suspension the bleaching of 1,3-diphenylisobenzofuran was followed. The quantification of the absorbance, α , in suspension was afforded by Amore *et al.* in standard 1 cm pathlength fluorescence cells, measuring the diffuse and total, R_t , reflectances and the diffuse transmittance, T , in a spectrophotometer fitted with an integrating sphere:

$$\alpha = 1 - R_t - T - L, \quad (9.4)$$

where L includes the radiation losses through the cell lateral walls, estimated for dilute suspensions in square cells as twice the measured diffuse

reflectance at the front face of the cell. Quantum yields were calculated in reference to a standard compound in solution. Assuming that the supporting material does not absorb light at the wavelengths of interest:

$$\Phi_{\Delta} = \Phi_{\Delta}^R \frac{r}{r_R} \frac{1 - 10^{-A_R}}{\alpha}, \quad (9.5)$$

where “R” refers to the reference standard, r is the rate of $^1\text{O}_2$ photogeneration measured by the consumption of a purely chemical scavenger and A_R is the absorbance of the reference solution. To stabilize aqueous suspensions during spectroscopic measurements 10% w/w polyvinyl alcohol was added. The validity of the method was tested by the comparison of the dye absorbance spectrum in solution and in suspension. For dilute suspensions, absorbances were calculated as:

$$A_{\text{susp}} = -\log(1 - \alpha). \quad (9.6)$$

Corrections should be made if the supporting material absorbs at the wavelengths of the experiment.⁴⁰ Results presented in Figure 9.3 clearly show the validity of the calculations.

Fullerenes and carbon nanotubes were considered as candidates for the generation of $^1\text{O}_2$ in solid matrices.⁴² The C_{60} triplet state was quenched by oxygen in the solid state when bound to a 98:2 styrene-divinylbenzene copolymer. The apparent quenching constant was two orders of magnitude lower than in benzene solution but swelling might render the system useful when suspended in appropriate solvents. The limitation of oxygen diffusion in the solid state and/or aggregation of C_{60} probably explain the difference of quenching constants.⁴³ C_{60} bound to an insoluble hydrophilic polymer based on Sephadex *G*-200 has been shown to generate $^1\text{O}_2$ by detection of its IR emission in aqueous suspensions.⁴⁴

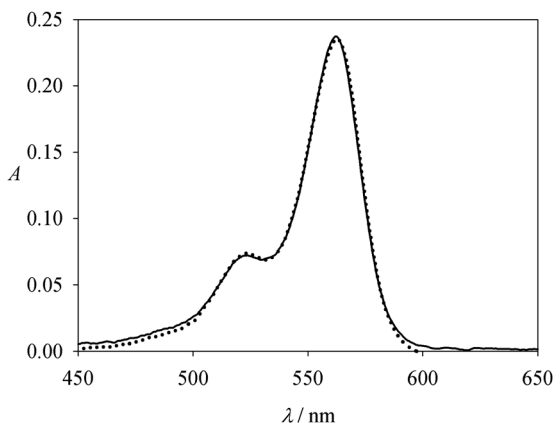


Figure 9.3. Rose bengal absorption spectrum in 3 mL 10% polyvinyl alcohol/water before (full line) and after (dotted line) adding 20 mg NovaSyn®. Reproduced from ref. 40.

Supported ruthenium complexes have been used as photooxidation sensitizers. Buell and Demas joined $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Ru}(\text{phen})_3]^{2+}$ (bpy = 2,2'-bipyridine; phen = 1,10-phenanthroline) by electrostatic binding to a Dowex 5OW-X1 cation-exchange resin with 4% charge coverage. No dye leakage in methanol or methanol/water suspension was observed.⁴⁵ The authors determined photooxidation quantum yields with the following approach. They prepared a slurry of the dye-resin complex in methanol mixed by a magnetic stirrer – the slurry was optically thick between 360 and 500 nm – and saturated it with O_2 before and during the course of the photolysis. The cell was irradiated with the 488 nm line of an argon ion laser, whose power, P , was corrected to obtain the effective power, P_{eff} , by the following equation:

$$P_{\text{eff}} = (1 - R_s)(1 - R)P, \quad (9.7)$$

where R is the diffuse reflectance and R_s is the specular reflectance for the air/glass/methanol interface, calculated using the Fresnel law. For the photooxidation of tetramethylethylene to its hydroperoxide, they obtained quantum yields as high as 0.77 with the $\text{Ru}(\text{bpy})$ -resin complex in methanol, as compared with 0.86 for rose bengal in methanol or methanol/water homogeneous solution and 0.19 for P-rose bengal (Sensitox from Hydron Laboratories) suspended in methanol. Measured yields are lower bounds to Φ_{Δ} . It should be borne in mind that P-rose bengal does not swell in alcohol.

Rose bengal was attached to photozymes, water-soluble polymers obtained by copolymerization of hydrophobic and hydrophilic units as poly(sodium styrenesulfonate-styrene-vinylbenzylchloride) and poly(sodium styrenesulfonate-2-vinylnaphthalene-vinylbenzylchloride). In aqueous solution, these polymers adopt a compact conformation, resulting in the formation of hydrophobic microdomains. Aside from microenvironmental effects determining the position of rose bengal bands, visible spectra for both polymers at dye concentrations of the order of 1% mol mol⁻¹ are very similar to that of the dye in ethanol (Figure 9.1), showing that rose bengal is essentially monomeric. Based on the consumption of 1,3-diphenylisobenzofuran in methanol in the zero-order regime, Φ_{Δ} values between 0.68 and 0.85 were obtained. Rose bengal in methanol solution was used as the standard. The $^1\text{O}_2$ scavenger is scarcely soluble in protic solvents but it is easily solubilized in the hydrophobic microdomains of photozymes. According to these quantum yield values, the authors conclude that no energy wasting through self-quenching or excimer formation takes place.⁴⁶ Nowakowska *et al.* attached rose bengal to water-soluble poly[(sodium *p*-styrenesulfonate)-*co*-(4-vinylbenzyl chloride)]. No dye aggregation was also found in this case; the repulsive interactions of negatively charged groups in most of the polymer units overcome the hydrophobic interaction among dye molecules.⁴⁷ For this polymer, based on the 1,3-diphenylisobenzofuran oxidation in methanol, the authors obtain $\Phi_{\Delta} = 0.73$, near the value found for rose bengal in the same solvent. Consumption of $^1\text{O}_2$ was not in the zero-order regime. The results depend in this case on the rate constant ratio k_v/k_{iv} (Scheme 9.1), taken from data obtained in methanol solution. However, this ratio can be quite different nearby the polymer,

where $^1\text{O}_2$ decays. Furthermore, interaction between the acceptor and the polymer may result in an error if the concentration in the bulk is considered. Therefore, the results have to be taken with caution.

The main concern about soluble polymeric photosensitizers regards their separation from the reaction medium after photooxidation has been carried out. Dialysis is a possible choice.⁴⁸ The change of pH is another possible way, as demonstrated by Ferrari *et al.* in their study of chitosan modified with rose bengal.⁴⁹ Resource can also be made to external stimuli such as temperature. A thermoresponsive polymer, poly(*N*-isopropylacrylamide), which has a suitable critical solution temperature (31 °C) and is soluble in water at or below room temperature, methanol and other solvents, has been copolymerized with 4% mol mol⁻¹ vinylbenzyl chloride and rose bengal has been attached to 96% of the vinylbenzyl units. Absorption spectra in methanol and water given in Figure 9.4 clearly show that the dye becomes aggregated in water. It is concluded that aggregation results from the formation of hydrophobic microdomains in water where the dye is located at a high local concentration. For the polymer in methanol $\Phi_{\Delta} = 0.55$ was obtained by 1,3-diphenylisobenzofuran oxidation, while in water $\Phi_{\Delta} = 0.24$ was measured by oxidation of anthracene-2-sulfonic acid sodium salt and compared with oxidation by rose bengal in a homogeneous solution.⁵⁰ Aside from aggregation and self-quenching, the effects of viscosity on the availability of O_2 at the dye location and solvent on the intersystem crossing quantum yield were considered. The viscosity reduces the apparent Φ_{Δ} only if O_2 availability is a limiting factor. The triplet quantum yield, Φ_{T} , of xanthene dyes increases in protic solvents;⁵¹ however, Φ_{Δ} decreases in water if the dye is confined in hydrophobic microdomains as assumed by the authors.

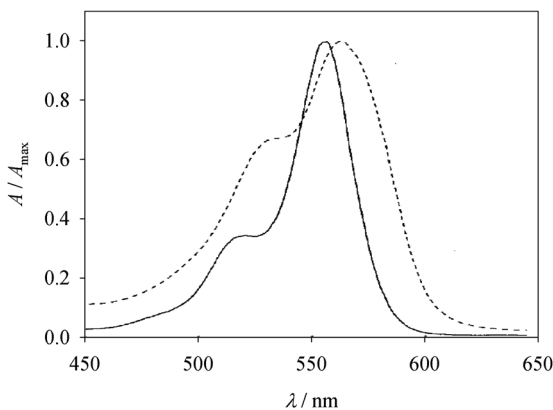


Figure 9.4. Absorption spectra of rose bengal attached to poly[(*N*-isopropylacrylamide)-*co*-(vinylbenzyl chloride)] in methanol (solid line) and water (dashed line). M. Nowakowska, M. Kępczyński and M. Dąbrowska, Polymeric Photosensitizers, 5. Synthesis and Photochemical Properties of Poly[(*N*-isopropylacrylamide)-*co*-(Vinylbenzyl Chloride)] Containing Covalently Bound Rose Bengal Chromophores, *Macromol. Chem. Phys.*, 2001, **202**, 1679–1688, Copyright Wiley-VCH Verlag GmbH & Co. KGaA, reproduced with permission.

The stability of insoluble $^1\text{O}_2$ sensitizers against light has been addressed by Oliveros *et al.*,⁵² who analyzed Sensitox and Sensitox II among other polymers. Aside from dye bleaching,¹⁶ the effect of $^1\text{O}_2$ on the polymeric backbone has to be considered.⁵³ A recent study showed that polystyrene and poly(phenylsilesquioxane) are among the most stable polymers, whereas poly(methyl methacrylate) has the lowest resistance among the studied polymers.⁵⁴

9.3. Photophysical Studies on Light-Scattering Materials

The aim of the summary given in Section 9.2, which is by no means comprehensive, was to give a brief account of the difficulties found in the quantification of the efficiency of heterogeneous photosensitizers to yield $^1\text{O}_2$. Obviously, the right figure of merit is Φ_Δ . Its quantification basically requires the knowledge of the rates at which light is absorbed and $^1\text{O}_2$ is produced and various steady-state and time-resolved methods are available for this purpose.²⁵ Two common methods to quantify $^1\text{O}_2$ production are (a) chemical trapping by a suitable $^1\text{O}_2$ scavenger and (b) measurement of any property linearly dependent on the $^1\text{O}_2$ concentration, *viz.* phosphorescence, and comparison with the same property of a suitable standard. Both methods were described by Scurlock *et al.* in their detailed study on the $^1\text{O}_2$ production by acridine in solid polystyrene.⁵⁵ The system was essentially transparent, allowing calculation of absorbance through the Beer–Lambert law.[§] A calibrated energy meter was used to quantify the incident light. Method (a) requires, among other factors, that physical quenching by the $^1\text{O}_2$ trap may be safely neglected and direct interaction between trap and photosensitizer may be excluded. Details are given in the same reference. Method (b) requires the existence of a suitable standard and matching geometries, and, if possible, identical absorbances between sample and standard. By laser time-resolved $^1\text{O}_2$ phosphorescence measurements, Φ_Δ can be evaluated obtaining the ratio of intensities at $t = 0$ or the ratio of integrated signals. In the second case, the signal can be expressed as:

$$I_\Delta = \kappa n^{-2} \Phi_\Delta k_r \tau_\Delta E_L (1 - 10^{-A}), \quad (9.8)$$

where κ is an instrumental constant, n is the refractive index of the medium at the phosphorescence wavelength, k_r and τ_Δ are the $^1\text{O}_2$ radiative rate constant and lifetime, respectively, E_L is the laser energy and A is the absorbance. While τ_Δ can be measured for the sample and the reference, evaluation of k_r ratios requires some explicit model.⁵⁵ Results obtained with both methods yield $\Phi_\Delta = 0.56 \pm 0.05$, near 70% of the value in toluene solution. The authors conclude that physical quenching of $^1\text{O}_2$ by rubrene, the acceptor used, is negligible in polystyrene, that all acridine triplets are quenched by molecular oxygen, and acridine excited singlets are not quenched. The causes for the

[§]A small contribution to the apparent absorbance due to light scattering was subtracted. This method works only if the light-scattering contribution is almost negligible.

lower Φ_{Δ} found in polystyrene may be a lower Φ_T value, an inhomogeneous distribution of dye molecules within the polymer matrix, a nonequilibrium distribution of molecular oxygen due to diffusional constraints, and depletion of oxygen by reaction with the matrix.

According to the preceding discussion, measurements on transparent solids do not differ essentially from those in liquid solutions but the quantity of unknowns may be larger on dealing with solids. Another point of interest is reabsorption of fluorescence (*vide infra*), a factor that would lead to an increase of Φ_{Δ} as compared with liquid solutions, where working absorbances are generally low. Indeed, fluorescence self-quenching might repopulate the triplet state, leading to higher apparent Φ_{Δ} values. In that case, studying the effect of dye concentration may be worthwhile. However, in the presented example, samples were 1 mm thick and their absorbance at 355 nm (wavelength at which acridine was excited) was *ca.* 0.7. Though some reabsorption may be present, the expectedly low fluorescence quantum yield, Φ_F , of acridine in the polymer may render this possibility as irrelevant.

Aside from the difficulties usually encountered in the determination of Φ_{Δ} for homogeneous solid solutions, for heterogeneous solids and suspensions light scattering is another complicating factor for the measurement of absorbances and calculation of absorption spectra. In general, the absorbance of a light-scattering sample should be calculated using eqn (9.4). If the sample is optically thick $T = 0$ and, if no lateral losses take place, $L = 0$. Normally, the last condition is fulfilled for an optically thick sample if its diameter is larger than its thickness, see Figure 9.5, which is normally the case if reflectance is measured in a spectrophotometer fitted with an integrating sphere accessory. In this case:

$$\alpha = 1 - R_t. \quad (9.9)$$

This is valid for solid samples but it should equally hold for optically thick slurries. For dry or wet solids that can be held in vertical position, measurements can be currently made in the capsules provided with the

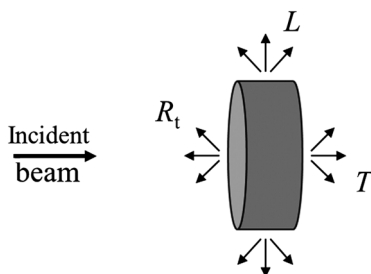


Figure 9.5. Total reflectance, R_t , transmittance, T , and lateral losses, L , for a light-scattering medium.

integrating sphere accessory. For slurries, round cells with optical windows can be used. Irradiation experiments have to be carried out using the same geometry as in reflectance measurements. It must be recalled that the presence of a solvent may increase the thickness needed to assure no transmittance, typically 1–3 mm for dry solids of micrometer particle size, as the refractive indexes of the solid and the liquid phase become closer. Diffuse transmittance should be measured to assure optical thickness. For optically thick samples in general, the absorption spectrum can be obtained measuring the diffuse reflectance and calculating the remission function, $F(R)$, through eqn (9.1). For the spectrum to be correctly obtained the sample should behave as an ideal, Lambertian scatterer³⁰ and the scattering coefficient, see eqn (9.2), should be constant within the wavelength interval of interest. According to our experience, these assumptions are in general good approximations.

Determination of absorptances for suspensions is in general more difficult because all terms in eqn (9.4) should be retained and diffuse transmittance and total reflectances should be measured simultaneously. Lateral losses can be reduced using thin cells. Suspensions may be stabilized with high viscosity additives like polyvinyl alcohol for aqueous or alcoholic suspensions. Eventually surfactants can be used. Again, irradiation experiments should be performed using the same geometry. For dilute suspensions, the absorption spectrum can be obtained from absorptances calculated using eqn (9.6). This is exemplified in Section 9.2, Figure 9.3.⁴⁰ For concentrated suspensions, more sophisticated methods as those developed in a series of papers by Gaigalas *et al.* (see ref. 56), requiring a spectrophotometer with the capability of introducing the measurement cell inside the integrating sphere and appealing to cumbersome calculations, should be used. No further details on these methods will be given here.

The problems usually encountered on determining the photophysical properties of light-scattering materials were recently reviewed by us, with emphasis in the work that our laboratory carries out since several years⁵⁷ and, therefore, only a brief summary will be given here. In most of these studies, microcrystalline cellulose was used as a model supporting material; various dyes were embedded through evaporation of their solutions in ethanol, a swelling solvent for cellulose, in which the microparticles were suspended. As a first example, a method to account for fluorescence quantum yields in this kind of materials will be presented. It is known that the measurement of reflectances from samples with $\Phi_F > ca. 0.2$ using integrating spheres can lead to severe errors because the detector cannot separate reflectance from luminescence. For optically thick samples, Mirenda *et al.*⁵⁸ developed a method that allows calculation of true reflectance spectra and absolute fluorescence quantum yields (no reference is needed) based on the measurement of reflectances without and with a suitable optical filter between the integrating sphere and the photomultiplier detector.

³⁰A Lambertian scatterer has the same apparent brightness independently of the view angle.

Vieira Ferreira *et al.*⁵⁹ arrived to similar results. Both quantities can be calculated from the following set of equations:⁵⁸

$$R_t(\lambda_0) = \frac{I(\lambda_0)R_{t,obs}^f(\lambda_0) - I^f(\lambda_0)R_{t,obs}(\lambda_0)}{I(\lambda_0) - I^f(\lambda_0)} \quad (9.10)$$

$$\Phi_{F,obs}(\lambda_0) = \frac{R_{t,obs}(\lambda_0) - R_{t,obs}^f(\lambda_0)}{I(\lambda_0)[1 - R_{t,obs}^f(\lambda_0)] - I^f(\lambda_0)[1 - R_{t,obs}(\lambda_0)]} \quad (9.11)$$

$$I(\lambda_0) = \int_{\lambda} f_{obs}(\lambda, \lambda_0) \frac{s(\lambda)}{s(\lambda_0)} \frac{\lambda_0}{\lambda} d\lambda \quad (9.12)$$

$$I^f(\lambda_0) = \int_{\lambda} f_{obs}(\lambda, \lambda_0) \frac{T(\lambda)}{T(\lambda_0)} \frac{s(\lambda)}{s(\lambda_0)} \frac{\lambda_0}{\lambda} d\lambda, \quad (9.13)$$

where $\Phi_{F,obs}(\lambda_0)$ is the observed fluorescence quantum yield (*vide infra*), dependent on the excitation wavelength λ_0 , $R_{t,obs}^f$ and $R_{t,obs}$ are the observed total reflectances measured with and without filter, R_t is the true total reflectance, $f_{obs}(\lambda)$ is the observed emission spectrum normalized to unit area, s the relative spectral responsivity of the detector, T the transmittance of the filter, and λ the emission wavelength. Diffuse reflectances may also be used instead of total reflectances. $\Phi_{F,obs}(\lambda_0)$ and $f_{obs}(\lambda, \lambda_0)$ differ from the quantities that would be measured at vanishingly low dye concentrations, Φ_F and $f(\lambda)$, because they are usually affected by fluorescence reabsorption and other factors. The method is particularly useful for materials having high $\Phi_{F,obs}$ values, which, in turn, can be used as reference for the relative measurement of light-scattering materials with lower quantum yields.

As the work of Schaap, Neckers and others demonstrates, see Section 9.2, one critical variable in the design of heterogeneous photosensitizers is the concentration of the dye. At low concentrations absorbance is low but at higher concentrations self-quenching takes place. Therefore, a compromise should be reached. From his results on CH_2Cl_2 soluble polystyrenes as a function of rose bengal concentration, Neckers concluded that “site/site” quenching takes place when dye molecules are closer than 50 styrene units.⁶⁰ This figure is equivalent to nearly 200 μg rose bengal per g of polymer or 7.5 nm between chromophores assuming homogeneous distribution. If these numbers are translated to P-rose bengal, strong fluorescence reabsorption must take place together with self quenching. In a previous paper, Paczkowski and Neckers discussed these features in detail, comparing the behavior of the same polymers when dissolved in CH_2Cl_2 and suspended in methanol, a solvent in which they are not soluble.⁶¹

With the aim of separating fluorescence reabsorption from other effects we developed a model that allows calculation of the true fluorescence spectrum,

devoid from reabsorption artifacts, from the observed spectrum. The model is valid for optically thick samples and leads to the following equations:⁶²

$$f(\lambda) = \frac{f_{\text{obs}}(\lambda, \lambda_0)}{\gamma(\lambda, \lambda_0)} \quad (9.14)$$

$$\gamma(\lambda, \lambda_0) = \frac{1}{1 + \sqrt{\frac{F(R)}{F(R) + 2}}} \times \frac{1}{1 + \sqrt{\frac{F(R)[F(R) + 2]}{F(R_0)[F(R_0) + 2]}}, \quad (9.15)$$

where $F(R)$ and $F(R_0)$ are the remission functions of the sample at the emission and excitation wavelengths, respectively. The function $\gamma(\lambda, \lambda_0)$ represents the probability that a photon emitted at wavelength λ escapes out of the sample. The same model allows the calculation of true fluorescence quantum yields from the observed values. At the same time, evaluation of remission function (*i.e.* absorption) spectra as a function of dye concentration allows calculation of the fraction of dye molecules in its monomeric form. In its simplest form, *i.e.* when the dye does not build up dimers or higher aggregates:

$$\Phi_F = \frac{\Phi_{\text{obs}}(\lambda_0)}{1 - P(\lambda_0)[1 - \Phi_{\text{obs}}(\lambda_0)]} \quad (9.16)$$

$$P(\lambda_0) = \int_{\lambda} f(\lambda)[1 - \gamma(\lambda, \lambda_0)]d\lambda. \quad (9.17)$$

The function $P(\lambda_0)$ can be interpreted as the probability that an emitted photon is reabsorbed. For pheophorbide *a* adsorbed on dry microcrystalline cellulose it was demonstrated that the dye remains in its monomeric form up to near $10 \mu\text{mol g}^{-1}$ of cellulose. Application of eqn (9.14) and (9.15) led to the results shown in Figure 9.6, showing that the fluorescence spectrum

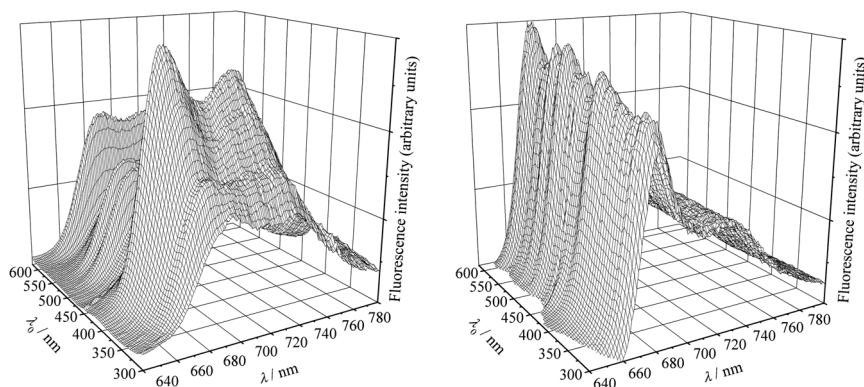


Figure 9.6. Uncorrected, $f_{\text{obs}}(\lambda, \lambda_0)$, (left) and corrected, $f(\lambda)$, spectra for pheophorbide *a* adsorbed on microcrystalline cellulose at $4.2 \mu\text{mol g}^{-1}$ of cellulose. Reproduced from ref. 63 with permission from the *Physical Chemistry Chemical Physics* Owner Societies.

is independent of excitation wavelength though experimental spectra show strong wavelength dependence due to reabsorption effects, whose magnitude depends on light penetration as a function of λ_0 . In spite of this, the corrected fluorescence quantum yield, Φ_F , obtained using eqn (9.16) and (9.17) decreased from 0.20 at 0.014 $\mu\text{mol g}^{-1}$ of cellulose to 0.06 at 8.9 $\mu\text{mol g}^{-1}$ of cellulose, while in ethanol $\Phi_F = 0.28$. The amplitude averaged fluorescence lifetime went from *ca.* 4 to *ca.* 2 ns in the same concentration interval, while the value found in ethanol is 5.9 ns. Though it is expected that Φ_F differs from the value found in solution due to interaction with the solid matrix, the variation with concentration can only be attributed to interchromophoric interactions, in spite of the fact that inspection of the absorption spectrum shows that the dye remains monomeric. The fact that quenching was dynamic led to the conclusion that energy migration and trapping takes place.⁶³

It is well known that the singlet excited state of dimers and higher aggregates formed at high dye local concentrations usually decay nonradiatively. This is particularly true for macrocyclic compounds like porphyrins and phthalocyanines devoid of bulky substituents. This is the case for ALTCPc on microcrystalline cellulose⁶² and silanized silica.⁶⁴ Aggregation is also the reason why Φ_A values in water suspension decrease rapidly with concentration in the last system. In contrast, fluorescence of dimers from rose bengal on microcrystalline cellulose has been found.⁶⁵ In some cases, as was observed for pheophorbide *a* (see last paragraph) and rhodamine 101 chemically linked to microcrystalline cellulose,⁶⁶ no evidence of dye aggregation was found. However, the lack of spectroscopic evidence does not preclude interaction among dye molecules leading to fluorescence quenching taking place. From studies carried out on systems composed by single dyes and pairs of dyes, it was shown that even though no noticeable interactions in the ground state take place, concentration quenching occurs when dye molecules are forced by the solid matrix to lay at close proximity. As a result, it was demonstrated that for xanthene dyes like rhodamines energy trapping takes place when dye molecules lay closer than 1.5 nm. Trapping may be static, when light is directly absorbed by traps, or dynamic, when excitation energy migrates from an excited monomer to a trapping center. Details on energy transfer and trapping models can be found elsewhere.^{57,67-69}

9.4. Triplet State and Singlet Oxygen Generation Quantum Yields and Decays

The obvious step toward a full characterization of the photophysics of dyes in heterogeneous light-scattering systems after the singlet state properties have been determined is the measurement of their triplet properties, namely quantum yield and decay. The determination of triplet decays can be easily performed by diffuse reflectance laser flash photolysis (DRLFP)⁷⁰ or time-resolved phosphorescence. Using the last technique we recently obtained triplet decays for eosin Y⁷¹ and Phloxine B⁷² on microcrystalline cellulose.

The compact structure of dry cellulose prevents the entrance of molecular oxygen to the matrix and, therefore, triplet decays may be measured without difficulty under air atmosphere. In both cases a concentration-independent bimodal distribution of lifetimes was obtained, showing that triplet–triplet and triplet–ground state interactions are negligible. Lifetimes are lower in cellulose amorphous domains and higher in crystalline environments. Φ_T values for dyes in light-scattering environments are far more difficult to obtain. Among the methods typically used to measure Φ_T in solution,⁷³ those based on quenching of singlet or triplet states require random distribution of quenchers, a condition difficult to guarantee in heterogeneous materials. Suitable reference compounds do not exist at all. EPR methods⁷⁴ require determination of light absorption by actinometry, which is complicated for light-scattering materials by the geometry of EPR cells. The interesting approach used by King *et al.*,⁷⁵ who measured the ground state recovery in the subnanosecond and millisecond time scales, was applied to low light-scattering polymer films and does not require quantification of triplet formation and absorbed photon rates. However, aside from the complexity of working simultaneously at quite different time scales, it would be difficult to apply to highly scattering materials.

We developed a method for the determination of Φ_T for optically thick samples based on light-induced optoacoustic spectroscopy (LIOAS).⁷⁶ Through a collinear arrangement, the sample contained in an *ad hoc* receptacle is irradiated from above with a pulsed laser beam and the optoacoustic signal is received by a PZT detector coupled to the opposite side through a thick quartz plate. Extreme care must be taken that no laser light reaches the detector, that no signal is produced through absorption of light by any system component different from the sample, and that good acoustic contact exists among sample, receptacle, quartz plate and detector. To assure the last condition the sample is pressed by a transparent window against the receptacle and other contacts are ensured with a thin layer of silicon grease. The following equation applies:^{||}

$$\frac{H}{E_L} = A(1 - R_t) \left(1 - \Phi_{F,obs} \frac{\langle \bar{\nu}_F \rangle}{\bar{\nu}_0} - \Phi_{T,obs} \frac{E_T}{hc\bar{\nu}_0} \right) \exp(-\mu d), \quad (9.18)$$

where H is the amplitude of the first maximum of the optoacoustic wave, E_L is the laser pulse energy, A is an instrumental constant, R_t is the total reflectance of the sample, $\langle \bar{\nu}_F \rangle = \int \bar{\nu}_F f(\bar{\nu}_F) d\bar{\nu}_F$ is the fluorescence average wavenumber, $\bar{\nu}_F$ being the fluorescence wavelength and $f(\bar{\nu}_F)$ the area-normalized fluorescence spectrum, $\bar{\nu}_0$ is the incident radiation wavenumber, E_T is the relaxed triplet energy, h is the Planck constant, c is the velocity of light in vacuum, μ is the Napierian sound absorption coefficient of the sample, and d its thickness. $\Phi_{T,obs}$ is used instead of Φ_T because fluorescence reabsorption

^{||}In eqn (2) of ref. 76 a phosphorescence term was incorrectly added. Phosphorescence is long lived and, therefore, delivered after the optoacoustic wave is fully developed.

may enhance triplet quantum yields. The thickness and compactness of all samples and calorimetric references (composed by a suitable dye in the same supporting material) should be held constant to ensure constancy of A and the exponential term throughout measurements. If these conditions are fulfilled, for long-lived triplets the origin and shape of the optoacoustic wave must remain constant.

Using the approach outlined in the last paragraph, measurements were performed for rose bengal and erythrosine B. Approximately constant quantum yields were obtained within the whole concentration range, 0.02 to 0.45 μmol dye per g of cellulose. For rose bengal, $\Phi_{\text{T,obs}} = 0.57 \pm 0.12$ and for erythrosine B, $\Phi_{\text{T,obs}} = 0.55 \pm 0.15$. To confirm these results and to increase the concentration range, $\Phi_{\text{T,obs}}$ was measured again for rose bengal using different techniques: LIOAS, DRLFP and laser-induced luminescence (LIL).⁷⁷ The last two methods yield only relative $\Phi_{\text{T,obs}}$ values but they are useful to establish their concentration dependence. As far as we know, this is the first time that DRLFP was used to calculate Φ_{T} values. The DRLFP signal:

$$S(t) = \frac{R_0 - R(t)}{R_0}, \quad (9.19)$$

where R_0 and $R(t)$ are the diffuse reflectances of the sample at the analyzing wavelength before and at time t after the laser pulse, respectively, is proportional to the number of molecules of the analyzed species, the triplet state in this case, at time t insofar $R_0 - R(t) < 0.1$.⁷⁸ On the other side, at vanishingly low laser energies the number of triplet molecules produced by the laser pulse is:

$$N_{\text{T}} = \frac{E_{\text{L}} \lambda_{\text{ex}}}{hc} (1 - R_{\text{t,ex}}) \Phi_{\text{T,obs}}, \quad (9.20)$$

where $R_{\text{t,ex}}$ is the total reflectance at the excitation wavelength, λ_{ex} , and the remaining symbols have the same meaning as in eqn (9.18). Therefore, $S(0)$ is proportional to the number of triplet molecules formed after the laser pulse. The representation of $S(0)$ as a function of E_{L} yields a function, whose slope at the origin is proportional to $\Phi_{\text{T,obs}} (1 - R_{\text{t,ex}})$, from which $\Phi_{\text{T,obs}}$ can be obtained. Extrapolated LIL signals (also proportional to the triplet number of molecules) at time zero can be handled in the same way. $\Phi_{\text{T,obs}}$ values at different rose bengal concentrations obtained by LIOAS are compared in Figure 9.7 with scaled $\Phi_{\text{T,obs}}$ values obtained by DRLFP and LIL and with scaled $\Phi_{\text{F,obs}}$ values, which were also obtained in ref. 77. Scaling is performed to match the absolute quantum yields obtained by LIOAS at the lowest concentrations. As the supporting material absorbs some radiation at $\lambda_{\text{ex}} = 532$ nm, $\Phi_{\text{T,obs}}$ and $\Phi_{\text{F,obs}}$ values are divided by the fraction of light absorbed by the dye, α_{ex} . Both $\Phi_{\text{T,obs}}/\alpha_{\text{ex}}$ and $\Phi_{\text{F,obs}}/\alpha_{\text{ex}}$ are slowly decreasing functions of the dye concentration. The decrease of $\Phi_{\text{F,obs}}/\alpha_{\text{ex}}$ was attributed mainly to dye aggregation and fluorescence reabsorption, though at low concentration dimers are fluorescent; at concentrations in excess of 0.4 $\mu\text{mol g}^{-1}$ of cellulose higher order – probably

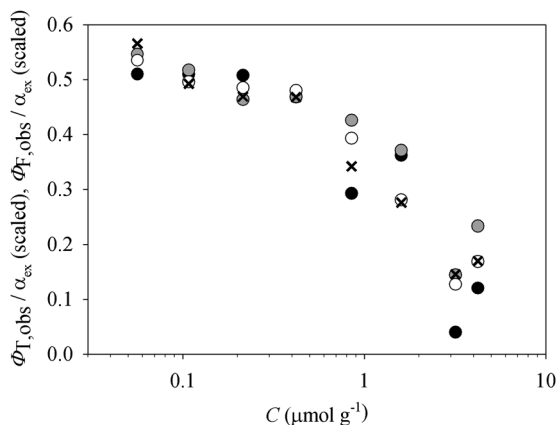


Figure 9.7. Triplet (LIOAS, black circles; DRLF, gray circles; LIL, white circles) and fluorescence quantum yields (crosshair) divided by α_{ex} (see text) for rose bengal adsorbed on microcrystalline cellulose as a function of the dye concentration. Reproduced with permission from Y. Litman *et al.*, *J. Phys. Chem A*, 2014, **118**, 10531, Copyright 2014 American Chemical Society.

dark – aggregates are formed,⁶⁵ leading to a parallel decrease of $\Phi_{F,obs}/\alpha_{ex}$ and $\Phi_{T,obs}/\alpha_{ex}$. It is shown that the practical concentration range for the generation of triplet states may be set around 0.1 to 0.4 $\mu\text{mol g}^{-1}$ of cellulose, the concentration range that maximizes $\Phi_{T,obs} \times (1 - R_{t,ex})$, reflecting the compromise between the decrease of $\Phi_{T,obs}$ and the increase of absorbance with rose bengal loading.

Several conclusions can be drawn from the behavior of rose bengal adsorbed on cellulose microparticles. Rose bengal is hydrophobic and cellulose is hydrophilic. The dye was included into the matrix by evaporation of ethanol, a solvent into which microcrystalline cellulose swells. In these conditions the dye is prone to aggregate and, indeed, more aggregates are formed at concentrations greater than 0.4 $\mu\text{mol g}^{-1}$ of cellulose; thereafter $\Phi_{T,obs}$ drops quickly. At this point, upon excitation at 532 nm, $R_{ex} \approx 0.5$, $\alpha_{ex} \approx 1$, and $\Phi_{T,obs} \approx 0.5$.⁷⁷ Cellulose samples are oxygen-free and, therefore, $^1\text{O}_2$ cannot be formed. A figure of merit for rose bengal in cellulose would be $g = \Phi_{T,obs} \times (1 - R_{ex}) \times \alpha_{ex} \approx 0.25$. Excitation at the absorption maximum, 563 nm, would yield $g \approx 0.3$ and, at 10-times larger concentration $g \approx 0.5$ would be obtained if aggregation remained at the same level (which should not be the case). For P-rose bengal, a system in which the dye is chemically bound to a hydrophobic matrix in a hydrophobic solvent excited at the absorption maximum at concentrations between 10 and 300 μmol rose bengal per g of polymer, considering $R_{ex} \approx 0$ ²³ and, as a working approximation, $\Phi_{T,obs} \approx \Phi_{\Delta} \approx 0.8$, the figure of merit would be $g \approx 0.8$, neglecting any absorption by the matrix. Two factors determine the difference: the apparently lower aggregation degree and a higher Φ_T for the monomeric dye in P-rose bengal. Chemical binding, essential to avoid dye leakage, does not necessarily hinder aggregation; more

effective is probably the compatibility between the dye, the polymeric matrix and the solvent. However, though measured Φ_{Δ} values do not depend on loading, it is not convenient to increase the dye loading arbitrarily because absorption saturates when R_{ex} approaches zero. Higher loadings increase the risk of dye bleaching and leakage.

Function g would be a good figure of merit only if most triplet states lead to the formation of $^1\text{O}_2$ and singlet state quenching can be neglected. In the latter case, $\Phi_{\text{T,obs}}$ represents an upper bound to Φ_{Δ} and g is the maximum value that may be attained. For fluorescent samples, experiments on fluorescence quenching by molecular oxygen are mandatory to assess the participation of the dye singlet state in the formation of $^1\text{O}_2$. Though various methods exist for the measurement of Φ_{Δ} in solution and, in general, in homogeneous transparent samples,²⁵ most of them cannot be applied to heterogeneous photosensitizers. Presently, one should rely on chemical quenching experiments and the question of how Φ_{Δ} can be measured with precision by other means remains momentarily unsolved. Even usage of LIOAS may yield doubtful results (*vide infra*). Care must be taken that no other processes involving the oxidation of the quencher by the dye excited states take place. Measurements should be performed in the zero-order limit both regarding chemical quencher concentration and oxygen pressure.

In an aerated liquid solution, the triplet state is rapidly quenched by molecular oxygen and following the $^1\text{O}_2$ luminescence at 1270 nm as a function of time after the triplet is completely quenched yields accurately the decay of $^1\text{O}_2$ and LIOAS can be used safely to measure Φ_{Δ} .⁷⁹ In glassy polymers, even if the triplet is the only quenched excited state, the situation is more complex.⁸⁰ As quenching by molecular oxygen is slower in that case because of diffusion constraints, the rates of formation and decay of $^1\text{O}_2$ may be similar and the luminescence time evolution results from the convolution of the decay functions of the dye excited state and $^1\text{O}_2$. In the extreme case that the excited-state decay is much slower, the luminescence decay will reflect the rate at which the excited state is depleted, yielding unusually long apparent $^1\text{O}_2$ lifetimes. Moreover, owing to the site dependence of excited state lifetimes, complex decays can be obtained. In homogeneous polymers, the triplet decay can be followed by flash photolysis and the $^1\text{O}_2$ decay function can be obtained by deconvolution. For polymer films the oxygen diffusion coefficients can be obtained by different techniques.⁸¹ For bead-like heterogeneous photosensitizers the characterization of oxygen transport is even more complex but the measurement of $^1\text{O}_2$ formation and decay by time-resolved $^1\text{O}_2$ luminescence as a function of oxygen pressure may yield relevant information. In principle, light scattering should not introduce severe difficulties into the determination. Though LIOAS can in principle be used to measure Φ_{Δ} , the determination makes sense only if the triplet state quenching is fast and the only source of slow heat release is $^1\text{O}_2$. Otherwise, dye triplets and $^1\text{O}_2$ may act as energy storing species. Photophysical studies as those outlined in Section 9.3 and in this section may help in answering questions such as the involvement of the dye singlet state in photosensitization, the role of dye-molecular oxygen

charge-transfer complexes, the existence of sensitizer-acceptor charge-transfer processes, and so on.

A last comment regards the fraction of the photogenerated $^1\text{O}_2$ molecules that are able to reach the target. The answer to this question is obviously complex and depends on the nature of the supporting material, the photosensitizer, the surrounding medium, and the acceptor. For polymeric swellable supports, compatibility among dye, support and solvent is essential to avoid dye aggregation. Furthermore, the acceptor has to be soluble in the liquid medium. Particle size and porosity are also relevant, mainly for inorganic supporting materials, in determining the surface area. Commonly, the acceptor will not be able to diffuse into the matrix and, therefore, reaction will be restricted to the surface of the photosensitizer or the nearby solvent volume. The root mean squared distance traveled by a diffusing molecule after time t in one dimension is given by the Einstein law:

$$x_{\text{RMS}} = (2Dt)^{1/2}, \quad (9.21)$$

where D is the $^1\text{O}_2$ diffusion coefficient. According to Redmond and Kochevar,⁸² this value amounts in water to 125 nm after one lifetime (4 μs , 37% $^1\text{O}_2$ remaining) and 220 nm after three lifetimes (5% remaining). These quantities should be multiplied by 4 in a solvent with similar D and 60 μs $^1\text{O}_2$ lifetime.

9.5. Conclusions

Heterogeneous photosensitized oxidations have long since captured the interest of the photochemical community. Though it was postulated by Kautsky early in the 1930s, the involvement of $^1\text{O}_2$ in this kind of processes was demonstrated by Foote and others only fifty years ago. Since then, a large number of materials with different degrees of efficiency have been developed. The activity in the field increased since the introduction of the concept of Green Chemistry. In spite of this, the battery of techniques needed to characterize the photophysics of heterogeneous photosensitizers is still insufficient. This is particularly true when heterogeneity of materials results in the occurrence of light scattering, to the point that no general methods exist to measure Φ_{Δ} in these conditions. Furthermore, much effort remains to be made in order to rationalize the design of heterogeneous photosensitizers and improve the availability of electronically excited species.

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Chapter 10

Production of Singlet Oxygen by Nanoparticle-Bound Photosensitizers

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10.1. Introduction

For PDT to be both effective and safe, it is very important to deliver the photosensitizer mainly to the target cells and not to nontarget cells. To improve this selectivity, a strategy is to use nanoparticles. Indeed, thanks to their size, nanoparticles allow selective accumulation of the PS in cancer cells, due to the enhanced permeability and retention effects of tumor tissues. Liposomes, micelles, polymer-based nanoparticles and dendrimers are nanostructures into which the PS is encapsulated and from which it is released into the target cells. After light excitation, the triplet PS can react with surrounding molecular oxygen by the type-1 or type-2 pathway in which singlet oxygen ($^1\text{O}_2$) is produced. These types of nanoparticles will not be described here. Instead, in this chapter we will focus specifically on nanoparticles possessing a three-dimensional rigid matrix, concentrating on the production of singlet oxygen ($^1\text{O}_2$) after excitation of PS that are coupled or encapsulated into these nanoparticles.^{1,2} With this type of nanoparticle, the mechanisms of PS action are slightly different from those described to date. The PS are not released into the target cells, but molecular oxygen has to diffuse into the nanoparticles and $^1\text{O}_2$ has to diffuse out of the nanoparticles. Different factors can influence the formation and the consumption of $^1\text{O}_2$ namely (i) oxygen access: the nanoparticles' matrix should be porous enough to be permeable to molecular oxygen as well as to $^1\text{O}_2$; (ii) $^1\text{O}_2$ can be quenched by the nanoparticle itself or by the surface agents before being able to reach the target cells. The chemical composition of the nanoparticle should be well defined. In other cases, for example in gold nanoparticles (AuNPs), the surface plasmonic effect enhances the photocurrent after light irradiation, which can lead to an AuNPs-PS energy transfer and an increase in $^1\text{O}_2$ formation; (iii) $^1\text{O}_2$ can react with the photosensitizer itself, leading to photobleaching; (iv) $^1\text{O}_2$ can be quenched by a nontarget substrate. This means that the localization of the nanoparticle is of great importance; (v) the effects of PS loading: most of the PS are hydrophobic and tend to aggregate. The amount of PS linked or encapsulated should be carefully controlled to avoid stacking of the PS that would lead to a decrease of $^1\text{O}_2$ formation.

Among the hundreds of papers describing the synthesis and characterization of nanoparticles and detection of $^1\text{O}_2$, we shall concentrate on just those papers whose authors compared the production of $^1\text{O}_2$ from the photosensitizer encapsulated or grafted into the nanoparticle with the production of $^1\text{O}_2$ of the same photosensitizers in solution. The aim of this is to better understand the influence of the encapsulation of the PS onto the $^1\text{O}_2$ production. Table 10.1 shows the papers we collected that describe the production of $^1\text{O}_2$ formed by photosensitizers encapsulated or covalently linked into inorganic nanoparticles. This does not, however, include quantum dots and all the nanoparticles that produce $^1\text{O}_2$ by themselves such as TiO_2 , ZnO and fullerene in which no PS have been encapsulated. Even if many authors discuss the formation of singlet oxygen by different techniques, there are in fact relatively few publications whose authors compare the $^1\text{O}_2$ formation of the PS

Table 10.1. Inorganic nanoparticles (except quantum dots, TiO₂, SiO₂) with encapsulated or grafted photosensitizers whose formation of ¹O₂ has been compared with the formation of ¹O₂ of the free photosensitizer.^a

Type of nanoparticles	PS	C/E	Phototoxicity test: <i>in vitro</i> model	Ref.	Detection of ¹ O ₂	PS-NP compared to PS
Gold	ZnPc	C	—	3	Time resolved luminescence	Increase
	ALA	E	Human neonatal dermal fibroblast and HT 1080	4	DCFH-DA	Increase
	Rose bengal	C	Cal-27	7	ABDA and H ₂ DCFDA	Increase
	5-ALA PpIX	C	Hela	5	H ₂ DCFDA	Increase
	Indocyanine green	C	A549	40	Direct detection	Increase
	Porphyrin-brucine	E	PE/CA-PJ34	41	<i>In vivo</i>	Increase
	Toluidine blue O	C	Bacteria	8	Direct detection	Increase
	ZnPc4	E	Hela	9	DMA	Same
	MB	E	HepG2	6	SOSG	Decrease
	Pc4	C/E	Hela	10	DPBF	Decrease
Graphene	MB	E	—	13	ABDA	Increase
	ZnPc	C	Hela, KB	14	DPBF	Decrease
	Ce6	E	Hela	15	DMA	Decrease
Mg–Al hydroxides	PpIX	E	—	16	Imidazole, 2,3-dimethyl-2-butene and linoleic acid	Increase
	ZnPc	C	QGY-7703, HeLa	17	DPBF	Increase
Iron oxide	Methylene blue	E	—	18	Direct detection DPBF	Decrease
	Purpurin-18 or HPPH	E	—	42	RNO	Increase
	Ce6	C	4T1	20	SOSG <i>in vivo</i>	—

Silica	<i>m</i> -THPC	E	—	22	ADPA	Increase
	Hypocrellin A	E	Hela	23 and 25	EPR and ADPA	Increase
	PpIX	C	—	24	DPBF and direct detection	Increase
	<i>Pc4</i>	E	A-375 and B16-F10	26	ABDA and EPR	Increase
	Hypocrellin A	E	Hela	24	ADPA	Increase
	Hematoporphyrin	C	HO-8910	29	ABDA	Increase
	PpIX	E	HCT 116 and HT29, A431, LLBC37, MDA-MB-231	27	APF kit (<i>in vivo</i>)	Increase
	HPPH	E	UCL-107, Hela	30	Direct detection and ADPA	Same
	PpIX	E	HCT 116	31	DPBF	Same
	TPC	C	MDA-MB-231	32	Direct detection	Same
	RB	E/C	MCF-7	33	SOSG	Same
	IP	C	Colon 26	34	Direct detection and ADPA	Same
	<i>Meso</i> -Tetra(<i>N</i> -methyl-4- pyridyl)porphine	E	SK-BR-3	39	Direct detection	Decrease
	TPP	E	—	35	AMDA + EPR	Decrease
	PpIX	C	Hela	36	Direct detection and DPBF	Decrease
	TPC	C	H1299	38	Direct detection	Decrease
	MB	E	C6	30	ADPA	Decrease
	Porphyrin, chlorin, <i>m</i> THPC	C	KB, A549, HUVEC	37	Direct detection	Decrease
	Chlorin e6	C	Hela	28	RNO	Decrease

^aPS: photosensitizer; C/E: C covalently coupled, E encapsulated; 4T1: murine breast cell line; A 375: human amelanotic melanoma cell line; A 431: epidermoid carcinoma cell line; A 549: human lung carcinoma malignant cell line; ABDA: 9,10-anthracenediyl-bis(methylene)dimalonic acid; ADPA: anthracene-9,10-dipropionic acid; ALA: aminolevulinic acid; AMDA: anthracene-9,10-bis-methylmalonate; APF: 2-[6-(4*V*-amino) phenoxy-3*H*-xanthen-3-on-9-yl] benzoic acid; B16-F10: Mouse melanoma cell line; C6: glial cell line; Cal-27: human oral squamous cell carcinoma; C6e: chlorin e6; DCFH-DA: dichlorodihydro-fluorescein diacetate; DMA: 9,10-dimethylanthracene; DPBF: 1,3-diphenylisobenzofuran; H₂DCFDA: 2', 7-dichlorodihydrofluorescein diacetate; EPR: electron paramagnetic resonance; H1299: lung cancer cell line; HCT 116: colon cancer cell line; Hela: human malignant cervical cells; HepG2: human hepatocellular liver carcinoma cell line; HO8910-PM cells: human ovarian cancer cell line; HPPH: 2-(1-hexyloxyethyl)-2-devinyl-pyropheophorbide; HT29: human colorectal adenocarcinoma cell line; HT 1080: human fibrosarcoma; IP: iodobenzylpyropheophorbide; KB: human nasopharyngeal epidermal carcinoma cell line; MB: methylene blue; LLBC37: lymphoblastoid cell line; MCF-7: squamous carcinoma 4451 cell lines, MCF-7; MDA-MB-231: breast cancer cells; *m*THPC: *meta*-tetra(hydroxyphenyl)chlorin; NP: nanoparticle; PAA: polyacrylic acid; *Pc4*: silicon phthalocyanine 4; PE/CA-PJ34: squamous cell carcinoma cells line; PpIX: protoporphyrin IX; QGY-7703: human Hepatoma cells; RB: rose bengal; RIF-1: Human Rap1-interacting protein; RNO: *p*-nitrosodimethylaniline; SK-BR-3: breast cancer cell lines; SOSG: singlet oxygen sensor green; TPC: 5-(4-carboxyphenyl)-10,15,20-triphenyl-chlorin; TPP: *meso*-tetraphenylporphyrin; UCL-107: human epithelial ovarian carcinoma; Zn*Pc*: zinc(II) phthalocyanine; Zn*Pc4*: zinc phthalocyanine; "—": not specified.

alone to the PS coupled to the nanoparticles. In the papers we analyzed the results by defining three types of nano-objects: nanoparticles in which the Φ_{Δ} of photosensitizers increases compared to free photosensitizers, nanoparticles in which the Φ_{Δ} of the encapsulated PS decreases compared to Φ_{Δ} of the free photosensitizer and systems in which Φ_{Δ} of the encapsulated or free PS are similar. We shall list the types of nanoparticles according to whether the photosensitizer is encapsulated (E) or covalently linked; whether *in vitro* or *in vivo* experiments were performed; whether singlet oxygen was detected by direct luminescence or by using chemical probes or if it was done *in vitro* or in cuvetts and, finally, according to the influence of encapsulation on Φ_{Δ} .

10.2. Gold Nanoparticles

Among all the metal nanoparticles, gold NPs (AuNPs) are receiving the most attention mainly due to their combination of unique properties that suit multiple applications such as labeling, delivery, heating and sensing. In most of the publications comparing the production of $^1\text{O}_2$ by photosensitizers coupled to AuNP or produced in solution, the authors detect an increase in the formation of $^1\text{O}_2$.³ For the first time in 2002, Russell's team compared free Pca and free Pc with TOAB (tetraoctylammonium bromide) phase-transfer reagent to the free Pc and Pc-coated gold nanoparticles with TOAB and found an increase of around 50% with the three-component Pc-coated gold nanoparticles. It is possible that TOAB affects both the excited singlet state of the free and bound Pc and the triplet energy transfer to molecular oxygen to form the excited singlet oxygen species. It is clear that three-component metal nanoparticles can generate $^1\text{O}_2$ with enhanced quantum yields when compared with free photosensitizers. Oo *et al.*⁴ also found a better production of reactive oxygen species (ROS) from 5-ALA-gold nanoparticles than 5-ALA alone on two different cell lines but an explanation for this was not given.

Benito *et al.*⁵ also developed gold nanoparticles coupled to 5-ALA. They observed an increase in ROS formation when compared with ALA alone and concluded that this may be due to a plasmon effect (no $^1\text{O}_2$ specific probe was used). The authors suggest that the surface plasmonic effect of AuNPs enhances the photocurrent between AuNPs and PpIX after light irradiation, which leads to an energy transfer to PpIX. The same conclusion was reached by Chu *et al.*⁶ They confined methylene blue in the close vicinity of an Au nanorod by incorporating it into SiO_2 during Au-core/ SiO_2 -shell nanoparticle (NP) growth to develop and produce a core-shell Au@ SiO_2 nanoparticle carrier. Using H_2DCFDA , after light irradiation the Au@(SiO_2 -MB) NPs were found to generate more hydroxyl radical and superoxide than both free MB and SiO_2 -MB NPs but free MB was found to produce the most $^1\text{O}_2$ among the three. The researchers also attributed this effect to the plasmonic enhancement effect of the Au core. After excitation, the intensified electromagnetic field in the proximity of Au was found to contribute to the increased absorption of photosensitizers. Two conditions are required here: (1) the excitation

energy of the PS should match the surface plasmon resonance energy of Au, and (2) the PS should be close to the Au. In the study by Wang,⁷ the increase observed could also have been due to enhanced absorption arising from the transverse surface plasmon resonance of NP.

Kuo *et al.*⁸ compared the luminescence at 1270 nm of ICG alone and Au PSMA (poly(styrene-*alt*maleic acid)-ICG) nanorods. The Φ_{Δ} of ICG and Au-PSMA-ICG nanorods were about 0.112 and 0.160, respectively. The authors found that the Au-PSMA-ICG nanorods generated more $^1\text{O}_2$ than ICG alone and this could be due to enhanced intersystem crossing, increased triplet yield of the photosensitizers, or the metal substrates, resulting in photostability for the photosensitizers. Nevertheless, it is not clear whether the detection at 1270 nm was due to $^1\text{O}_2$ or to ICG fluorescence.

Most of the studies describe an enhancement of $^1\text{O}_2$ formation but Shang *et al.*⁹ did not observe any difference between $^1\text{O}_2$ formation of loaded or free ZnPc, and Cheng *et al.*¹⁰ even detected a decrease in $^1\text{O}_2$ formation. In the paper by Shang *et al.* ZnPc is encapsulated into a nanogel composed of a PEGMA monolayer on the surface of gold nanorods and crosslinked *N*-iso-propylacrylamide (NIPAAm) and poly-(ethyleneglycol)-methacrylate (PEGMA). No difference was found between the $^1\text{O}_2$ formation with ZnPc alone or ZnPc loaded into the nanoparticles. Cheng *et al.*¹⁰ elaborated PEGylated AuNP-Pc and found $\Phi_{\Delta} = 50\%$ for free Pc in ethanol using DPBF, whereas $\Phi_{\Delta} = 35\%$ was estimated for ZnPc-PEGylated AuNPs.

10.3. Carbon Nanotubes

Single (SWNTs) or multiwalled carbon nanotubes (MWNTs) can be used as delivery agents for PDT photosensitizers. The influence of grafting or encapsulating photosensitizers on carbon nanotubes is difficult to evaluate, a point illustrated by the fact that only those publications that describe $^1\text{O}_2$ formation use aptamer¹¹ to control $^1\text{O}_2$ formation or the release of the photosensitizers,¹² which means it is impossible to run a comparison with free photosensitizer.

10.4. Graphene

Graphene is a two-dimensional material with sp^2 -bonded carbon atoms packed into a honeycomb lattice whose electronic, optical and structural properties make it suitable for applications in many fields like electronics, catalysis, gas storage and medicine. Wojtoniszak *et al.*¹³ developed graphene oxide functionalized with methylene blue. They used a chemical probe ABDA (9,10-anthracenediyl-bis(methylene)dimalonic acid) and observed a better $^1\text{O}_2$ formation for grafted methylene blue than free methylene blue in solution. In this case, the researchers suggest that adsorption of methylene blue on graphene oxide increased the yield of intersystem crossing, which gives enhanced $^1\text{O}_2$ quantum yield by the nanoparticles.

Conversely, two other studies reveal a decrease in $^1\text{O}_2$ formation; Wang *et al.*¹⁴ developed a core-shell multifunctional nanocomposite surrounded by polyethylene glycol and loading Zn-phthalocyanine (ZnPc). Using DPBF, they found that $^1\text{O}_2$ formation was weaker for the nanoparticles than for free ZnPc because of nanographene oxide's quenching effect. Li *et al.*¹⁵ synthesized hyaluronic-graphene oxide conjugates with chlorin e6. They used DMA to demonstrate that free Ce6 presents better $^1\text{O}_2$ formation than grafted Ce6 in water, illustrating the firm adsorption of the photosensitizer onto the graphene that results in quenching.

10.5. Mg-Al Hydroxides

Layered double hydroxides (LDHs) are synthetic clay materials that form successive layers of metal hydroxides separated by layers of anions and water. Kantonis *et al.* immobilized PpIX on layered double hydroxides and also synthesized a nanohybrid of LDH coupled to perfluoroheptanoic acid (LDH-PFHA) to increase the solubility of molecular O_2 . The reaction rate for the oxidation of three substrates was determined using imidazole, 2,3-dimethyl-2-butene and linoleic acid. Using imidazole, the immobilized PpIX was able to produce $^1\text{O}_2$ ¹⁶ five to nine times faster with free PpIX compared to LDH-PpIX and LDH-PpIX-PFHA, respectively. In contrast, with hydrophobic substrates LDH-PpIX-PFHA was found to give the fastest reactions. This was particularly noteworthy in the case of linoleic acid where the reaction with LDH-PpIX-PFHA as catalyst was four to seven times faster than with LDH-PpIX and free PpIX, respectively.

10.6. Iron Oxide with Polyacrylamide or Silica

For this type of nanoparticle, one paper describes an increase of $^1\text{O}_2$ from the PS into the nanoparticles and one paper a decrease of $^1\text{O}_2$ formation when the PS is linked to the nano-object.

Wang *et al.*¹⁷ developed fluorescent and mesoporous core-shell-structured nanoparticles and observed that the AlC_4Pc -incorporated $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{meso-SiO}_2$ particles exhibited much higher activity in photo-oxidation DPBF than the same amount of free AlC_4Pc in solution. They suggest that the mesoporous silica nanovehicle acts both as a carrier for AlC_4Pc and also as a nanoreactor to facilitate the photo-oxidation reaction.

Tada *et al.*¹⁸ elaborated silica-coated magnetic particles containing methylene blue (MagMB). The lifetime of $^1\text{O}_2$ was determined in CH_3CN (52 μs), and in water (3 μs); Φ_Δ of MagMB particles was estimated to be 0.03 ± 0.02 , while Φ_Δ of MB in CH_3CN is 0.50. According to the authors and reference,¹⁹ this decrease in $^1\text{O}_2$ production when MB is encapsulated in nanoparticles may be due to the scattering of the nanoparticles, the local sequestration of generated $^1\text{O}_2$ by the nanoparticle matrix, or an intrinsic lower encapsulated MB Φ_Δ .

10.7. Iron Oxide

Li *et al.*²⁰ developed PEG-functionalized iron-oxide nanoclusters loaded with chlorin e6. The authors compared $^1\text{O}_2$ generation for free Ce6 with encapsulated Ce6, but in their experiment the excitation was carried out at 704 nm where Ce6 does not absorb, which makes it difficult to draw any conclusions regarding the influence of encapsulation on $^1\text{O}_2$ generation. Nevertheless, ION-CePEGCe6 offered a good level of $^1\text{O}_2$ generation under 704 nm excitation.

10.8. Silica

Silica-based nanomaterials have emerged as promising vectors for PDT mainly because they are chemically inert and the silica matrix porosity is not susceptible to swelling or changes because of a varying pH. The particle size, shape, porosity and monodispersibility can be easily controlled during their preparation. Moreover, $^1\text{O}_2$ generated by the photosensitizer immobilized in the silica matrix can diffuse efficiently through the solution.

Of the publications studied, 7 papers described an increase of $^1\text{O}_2$ from the PS into the nanoparticles, 5 did not detect any significant difference and 7 related a decrease of $^1\text{O}_2$ formation when it is encapsulated into silica nanoparticles.

Rossi *et al.*²¹ developed protoporphyrin IX nanoparticle carrier. The quantum yield of free protoporphyrin solution ($\Phi_{\Delta} = 0.77$ in CCl_4 ; $\Phi_{\Delta} = 0.60$ in aqueous/TX100 solution) was found to be less than 0.9 for the porphyrin immobilized in silica spheres. The authors suggest that this increase could be due to the decrease in the monomer–dimer equilibrium because the photosensitizer is covalently attached to the matrix. Aggregation is indeed also known to reduce the quantum yield and lifetime of the excited triplet state of porphyrins and logically should consequently therefore also reduce the $^1\text{O}_2$ generation yield. Yan and Kopelman²² compared the photophysical properties of and the pH influence on free *m*-THPC with those of *m*-THPC embedded in silica nanoparticles. They used ADPA (anthracene-9,10-dipropionic acid) to determine that the exact reaction rate constant for free *m*-THPC and *m*-THPC embedded in silica NP is $2.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $4.8 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, suggesting that $^1\text{O}_2$ production from *m*-THPC embedded in silica NP exceeds that of free *m*-THPC.

Zhou *et al.* embedded hypocrellin A (HA) into porous hollow silica nanospheres.^{23,24} The same ESR signal was observed in an aqueous solution for HA free molecules or HA-NP, but the signal intensity was found to be smaller, implying that HA-NP could have a better $^1\text{O}_2$ generation ability than free HA. $\Phi_{\Delta} = 1.16$ was determined with ADPA taking free HA as a reference. They claimed that the increased $^1\text{O}_2$ generation ability of AM-HA as compared with free HA was possibly because the nanoparticle formation protected the interior HA molecules from exposure to the aqueous environment, thus slowing down the $^1\text{O}_2$ quenching speed.

Other silica nanovehicles with embedded hypocrellin A were synthesized two years later by Zhou *et al.*^{24,25} In this case, $\Phi_{\Delta} = 2.13$ taking free HA as a reference and defining its $\Phi_{\Delta} = 1$ at this experiment condition. The authors suggest that the presence of the silica nanovehicle significantly increases the ability of HA to generate $^1\text{O}_2$ because the nanovehicle protected and retained the long-lived singlet state of HA. Zhao *et al.*²⁶ encapsulated Pc4 using silica nanoparticles (Pc4SNP). Using ABDA as a $^1\text{O}_2$ chemical probe, they showed that for the Pc4 sample, the photobleaching rate caused by $^1\text{O}_2$ was much lower than that of Pc4SNP. Encapsulation of Pc4 into silicon nanoparticles improved $^1\text{O}_2$ production while decreasing photobleaching and aggregation.

Simon *et al.*²⁷ described the synthesis and photophysical studies of protoporphyrin IX (Pp IX) silica nanoparticles. ROS detection *in vivo* was performed using an APF (2-[6-(4'-amino) phenoxy-3*H*-xanthen-3-on-9-yl] benzoic acid) kit that is not specific to $^1\text{O}_2$. They evaluated the ROS generation of Pp IX silica nanoparticles and free Pp IX and quantification of cell internalization using HCT 116 and HT-29 colon cancer cells and demonstrated that ROS generation was significantly improved in the presence of PpIX silica nanoparticles in both cell lines.

Wang *et al.*²⁸ loaded chlorin e6 onto NaFY₄-based UCNP functionalized with polyethylene glycol to elaborate a UCNP-Ce6 complex. They observed significant production of $^1\text{O}_2$ after irradiation at 980 nm of UCNP-Ce6. In marked contrast, they noted that plain UCNP and free Ce6 at the same respective concentrations generated $^1\text{O}_2$ under the 980 nm light, due to the fact that Ce6 does not absorb at this wavelength.

Zhang *et al.*²⁹ compared the photo-oxidation effect of SiO₂@hematoporphyrin (HP) nanocomposites and the same numbers of HP molecules in a homogeneous solution. SiO₂@HP nanocomposites produce 10–15 times more $^1\text{O}_2$ than free HP does in a homogeneous solution. The authors suggest that HP molecules are located in the nanocomposites that results in a higher local concentration in the mesopore than in the homogeneous solution. Moreover, the mesoporous structure's high surface volume allows the absorption of ABMD into the pores, thus increasing the local concentration of ABMD and the efficiency of the reaction between the probe and $^1\text{O}_2$. During irradiation, the endoperoxide formed leads to a concentration gradient between the mesoporous and solution phases that acts as a driving force for voluntary exchange of ABMD and the photo-oxidized product between two phases until the photo-oxidation reaction is complete. In SiO₂@HP, a shorter diffusion time of $^1\text{O}_2$ is required before photo-oxidation processes because $^1\text{O}_2$ generation and photo-oxidation occur in the mesoporous channel. HP molecules are bound to the mesopores that are made up of inert silica matrix and are therefore less reactive than water which means that the singlet and triplet excited state of HP molecules are possibly therefore less quenched or deactivated. The degree of freedom of the HP molecules reduced by this microenvironment of the silica mesopore causes a longer excited-state lifetime and an enhancement of $^1\text{O}_2$ generation efficiency. The consequence of all these

factors is that the photo-oxidation efficiency of the mesoporous materials is higher than the homogeneous one.

In 2003, the team of Prasad³⁰ synthesized ultrafine organically modified silica (ORMOSIL) based nanoparticles entrapping HPPH. They used ADPA to compare the $^1\text{O}_2$ generation from free HPPH and HPPH solubilized in micelles or doped in nanoparticles. Similar efficiencies of $^1\text{O}_2$ generation were found in both cases. It should be noted that the absorption spectra of HPPH were also similar for both. In 2010, Thienot *et al.*³¹ described one-pot synthesis of a very stable hybrid versatile nanocarrier made of PpIX silica-based nanocarriers with a bilayer coating of triethoxyvinylsilane. The authors compared the PpIX silica-based nanoparticles in PBS with PpIX in DMF. Similar levels of effectiveness were observed in both cases although it is difficult to draw conclusions because the solvents are different. Our team also conjugated a 5-(4-carboxyphenyl)-10,15,20-triphenyl-chlorin to a hybrid gadolinium oxide nanoparticle coupled to peptides to target neuropilin-1.³² TPC-grafted within the nanoparticle matrix was found to present the same quantum yield of $^1\text{O}_2$ as free TPC in a solution, that is to say that the photosensitizer was mainly in a monomeric form without aggregation inside the NPs. In a similar way,³³ rose bengal was conjugated to organically modified silica nanoparticles by electrostatic and covalent interaction. The relative $^1\text{O}_2$ generation yield of RB and its complexes with SiNP did not reveal any significant differences.

Prasad's team³⁴ reported new ORMOSIL with HPPH linked to the nanoparticles instead of being physically entrapped. They first synthesized iodobenzyl-pyrosilane (IPS) that is a precursor for ORMOSIL with the grafted iodobenzylpyropheophorbide (IP). Different formulations were synthesized by changing the IPS/VTES (vinyltriethoxysilane) ratio to study the influence of loading on the photophysical properties of the pyropheophorbide. IP incorporated within nanoparticles is capable of generating $^1\text{O}_2$ with a yield comparable to that of $^1\text{O}_2$ generated by IP/Tween-80 micelles using both direct detection of $^1\text{O}_2$ luminescence and ADPA. The $^1\text{O}_2$ generated by PS (IP) in surfactant (Tween-80) micelles depends on the relative amount of surfactant that protects hydrophobic PS molecules from aggregation. The $^1\text{O}_2$ generated within nanoparticles is mostly deactivated outside nanoparticles that leads to bleaching of ADPA. IP/Tween-80 micellar suspension demonstrated higher $^1\text{O}_2$ generation than the nanoparticles whatever the loads on the photosensitizers.

Li *et al.*³⁵ developed pH-responsive silica nanoparticles for controllable $^1\text{O}_2$ generation. By a spin-trapping technique using 2,2,6,6-tetramethyl-4-piperidone (TEMP) as the spin-trapping agent, the authors showed that TPP-NP produced a similar electron paramagnetic resonance signal, indicating the generation of $^1\text{O}_2$. In the case of TPP or TPP-NP, the photobleaching of AMDA was found to be superior with free TPP than with TPP-NP. Tu *et al.*³⁶ also reported the elaboration of mesoporous silica nanoparticles conjugated with PpIX *via* the amine group of 3-aminopropyltrimethoxysilane. The absorption decay curves of DPBF were found to be faster for free PpIX than for covalently linked PpIX. Direct luminescence at 1270 nm in acetone was also performed. Tang *et al.*¹⁹ also observed a decrease of Φ_Δ after encapsulation of methylene

blue in polyacrylamide-based nanoparticles. They showed that the delivery of $^1\text{O}_2$ was reduced to varying degrees by encapsulation. This difference in activity was attributed to the various microenvironments in the nanoparticles, in particular, the local sequestration of produced $^1\text{O}_2$ by the matrixes or even the dimerization of MB, which occurred to different extents in the matrixes. Nevertheless, they also showed that using nanoparticles can prevent the embedded MB from being reduced by diaphorase enzymes, thereby retaining the photoactive form of MB for efficient PDT treatment.

Selvestrel *et al.*³⁷ synthesized ORMOSIL nanoparticles with alkoxy-silane-porphyrin, alkoxy-silane *m*-THPC like chlorin and an alkoxy-silane *m*-THPC like chlorin attached to the nanoparticles with 4 silane groups. They measured the Φ_Δ in MeOD or D_2O . Whereas Φ_Δ of *m*-THPC in MeOD is 0.71, they found measurements of between 0.55 and 0.67 for the two nanoparticles. In deuterated water, $^1\text{O}_2$ production of nanoparticles was found to be very similar to that of *m*-THPC but the $^1\text{O}_2$ production of the nanoparticle doped with the alkoxy-silane *m*-THPC like chlorin attached to the nanoparticles with 4 silane groups was much lower. The authors suggest that water penetrating the nanoparticle may lead to distortion of the tetracoordinated chlorin and that this would increase the rate of internal conversion and a decrease of $^1\text{O}_2$ production. This is due to the fact that the chlorin is rigidly attached to the silica matrix and cannot compensate by conformational realignment.

To evaluate the influence of loading onto the Φ_Δ and the Φ_f , our team³⁸ synthesized silica nanoparticles with a Gd_2O_3 - Tb_2O_3 core surrounded by a polysiloxane shell in which is covalently bound different amounts of tetraphenyl monocarboxylic chlorin. We were able to show that the amount of covalently grafted PS has a slightly negative influence on Φ_Δ and Φ_f . Indeed, when the grafting was too high, we observed a decrease of Φ_Δ compared to the one obtained using the same amount of chlorin in solution due to both FRET and partial quenching linked to the formation of dimers.

A final interesting example is the work of Li *et al.*³⁹ who synthesized base silica nanoparticle-attached *meso*-tetra(*N*-methyl-4pyridyl)porphyrin (SiO_2 -TMPyP) for pH-controllable photosensitization. The concept of this structure is that SiO_2 nanoparticles are triplet quenchers of the photosensitizer and/or $^1\text{O}_2$ at alkaline pH. In weak acidic solutions, the photosensitizer is released from the nanoparticle and can then produce $^1\text{O}_2$. By monitoring $^1\text{O}_2$ luminescence, Φ_Δ were found to be pH dependent, dropping from ~ 0.45 in a pH range of 3–6 to 0.08 at pH 8–9. (For free TMPyP $\Phi_\Delta = 0.58$ at pH 7.35.) The lower Φ_Δ values obtained from SiO_2 -TMPyP relative to those from free TMPyP in acidic solutions might be due to aggregation of TMPyP and quenching of triplet TMPyP and/or $^1\text{O}_2$ by SiO_2 nanoparticles.

10.9. Conclusion and Perspectives

In conclusion, it is worthwhile analyzing the results in terms of the factors that can influence Φ_Δ such as oxygen access, quenching of $^1\text{O}_2$ by the nanoparticle itself and the effects of PS loading or nanoparticle surface modification

because for each type of nanoparticle, an increase or decrease of Φ_{Δ} could be observed. In fact, there are few publications whose authors compare the $^1\text{O}_2$ formation by the photosensitizer alone to that by the photosensitizer coupled to the nanoparticles and therefore it is difficult to draw a general conclusion. For gold nanoparticles, it seems that the surface plasmonic effect of AuNPs could enhance Φ_{Δ} . With silica nanoparticles, the diffusion of the O_2 and of the chemical probe into the silica shell or the pores could increase the local concentration and efficiency of the reaction between the probe and also $^1\text{O}_2$ is improved. For graphene, authors have claimed that the adsorption of methylene blue on graphene oxide increased the yield of intersystem crossing that leads to enhanced $^1\text{O}_2$ quantum yield by the nanoparticles. In general, nanovehicles can protect the photosensitizer (photobleaching) and act as a nanoreactor and appropriate loading also results in a decrease of aggregation. In the future, it might be useful to design experiments to evaluate the influence of each parameter on the formation and consumption of $^1\text{O}_2$. Parameters such as chemical composition of the nanoparticles, size of the nanoparticles, PS loading could be taken into account to evaluate the benefit of the encapsulation/grafting onto nanoparticles for the formation and consumption of $^1\text{O}_2$ compared to free photosensitizers.

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Chapter 11

Endogenous Singlet Oxygen Photosensitizers in Mammalians

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11.1. Introduction

The usual mechanism of photodynamic generation of $^1\text{O}_2$ requires the absorption of optical radiation in dye molecules (photosensitizers), which transfer energy to molecular oxygen. This reaction may occur in any environment, in which a photosensitizer, oxygen, and radiation are present at the same time. This includes of course living organisms like single cells, plants, or mammals.

In daily life, mammals are frequently exposed to different types of optical radiation that origin from artificial light sources and solar radiation. Together with the fact that mammals possess sufficient amounts of oxygen leaves the question of potential photosensitizers. Mammals consist of a huge number of diverse cells, which in turn comprise diverse biomolecules such as proteins, amino acids, lipids, fatty acids, vitamins, DNA and others. These biomolecules serve for different purposes and appear in different cells in different concentrations. Depending on their chemical structure, some of these molecules may have the potential to serve as endogenous photosensitizers.

On the Earth's surface, mammals are exposed to radiation that ranges from the ultraviolet (UV) to the infrared (IR) spectrum. Thus, cellular components can absorb radiation of that broad spectral range. However, most of the scientific publications report on $^1\text{O}_2$ -mediated cell effects, which are caused by UV radiation with wavelengths from 280 to 400 nm.¹⁻⁵ This is due to the fact that most of the endogenous photosensitizers show a high absorption coefficient in the UV. Some information is currently available whether and to what extent visible light induces $^1\text{O}_2$ generation in mammals.⁶⁻⁸ Infrared radiation may damage cellular structures and affect skin integrity *via* mechanisms similar to ultraviolet radiation but obviously without the involvement of $^1\text{O}_2$.⁹

11.2. $^1\text{O}_2$ -Mediated Processes in Mammalian Cells

UVA is the most frequently reported spectral range of radiation, which is involved in the generation of $^1\text{O}_2$ and the subsequent cellular effects *in vitro* and *in vivo*.¹⁰⁻¹⁴ On the one hand, this reactive intermediate is responsible for triggering various cellular reactions *via* peroxidation of membrane lipids. On the other hand, the production of $^1\text{O}_2$ may lead to UVA-induced necrotic cell death.¹⁵

Oxidized membrane lipids like ceramides and lipid peroxides may trigger an intermediate signaling generating various protein kinases like mitogen-activated protein (MAP) and extracellular signal-regulated kinase (ERK).¹⁶ As a consequence, transcription factors are activated leading to the activation of heme oxygenase 1 (HO-1) gene expression.¹⁵ Similar data concerning $^1\text{O}_2$ involvement also exist for the collagenase (matrix metalloproteinase 1, MMP-1) gene, whose protein may be directly involved in skin photoaging.¹⁷

Moreover, $^1\text{O}_2$ exhibits a specific reactivity toward guanine yielding the main $^1\text{O}_2$ guanine oxidation product 8-oxo-7,8-dihydro-2'-deoxyguanosine in both isolated and cellular DNA.¹⁸ The formation of this product may lead to DNA misreplication, resulting in mutation, particularly G to T transversions.¹⁹ A study on the mutagenic specificity of solar radiation has indicated that not only UVB, but also UVA, participates in solar mutagenesis.²⁰ $^1\text{O}_2$, generated *via* endogenous photosensitizers like porphyrins and flavins, is exclusively responsible for the deleterious 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxoG) that is a product of a reaction of $^1\text{O}_2$ with the guanine moiety of cellular DNA.²¹

There is evidence that UVA-induced $^1\text{O}_2$ plays an important role in the pathogenesis of photodermatoses such as polymorphous light eruption as well as photoaging.¹³ The role of photosensitized production of $^1\text{O}_2$ was recently reviewed and the authors stated that UVA elicits a biologically significant number of cyclobutane pyrimidine dimers (CPDs) and 8oxoG at environmentally relevant doses.²² UVA is a relatively weak mutagenic, in agreement with a low induction of DNA damage. In contrast to CPDs, which may persist, 8oxoG is rapidly and efficiently repaired and contributes very poorly to solar UV mutagenesis. However, UVA could be involved in the initiation of skin cancers in the human population, at least in the case of heavy exposure to UVA, *i.e.* repeated and excessive use of sunbeds and sunlamps.

Besides damaging effects in skin, the photosensitized production of $^1\text{O}_2$ plays a role in a frequently occurring eye disease. Cataract is a significant cause of visual disability with relatively high incidence. Various *in vitro* and *in vivo* studies strongly support the hypothesis that light penetration into the eye is a significant contributive factor in the genesis of cataracts. The major effect is through photochemical generation of reactive oxygen species like $^1\text{O}_2$ and consequent oxidative stress to the tissue.¹¹

11.3. Absorption of Radiation

Radiation emitted by artificial light sources or solar radiation almost exclusively reaches the skin, the mucosa, and the eyes of mammals. In the case of solar radiation, wavelengths less than 280 nm are fortunately blocked by the ozone layer and cannot reach Earth's surface. However, the remaining ultraviolet radiation, UVB (280–320 nm) and UVA (320–400 nm), may reach the surface of mammals. Except for some medical treatments, artificial light sources emit visible and infrared radiation only.

The tissue and cells of mammals mainly comprise of water. For wavelengths longer than about 1500 nm, the IR radiation is almost completely absorbed in a 1 mm aqueous layer. This thickness should be equal to cells *in vitro* in cell culture medium (Figure 11.1). Infrared radiation with a wavelength longer than about 1500 nm penetrates mammalian tissue to a small extent due to radiation absorption that is enhanced by photon scattering. Consequently, radiation in the spectral range from 280 to 1500 nm reaches more likely endogenous photosensitizer inside mammalian tissue (*e.g.* skin, eye).

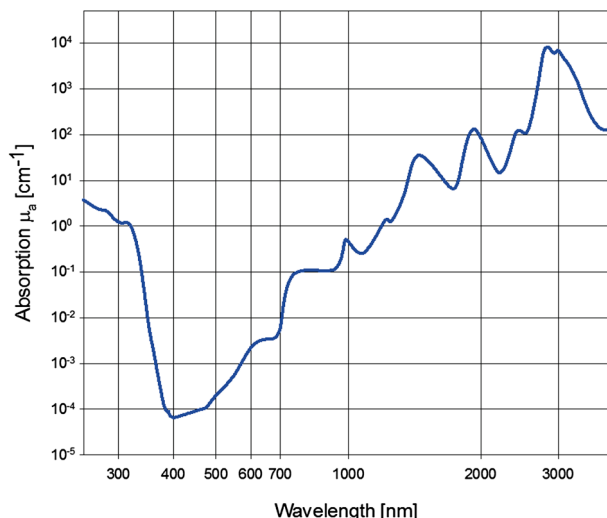


Figure 11.1. The absorption coefficient of water is shown in the range from 250 to 4000 nm.

The interaction of light with mammalian cells or tissue (in particular skin cells/tissue) has been mainly concerned with the effects of UVB (290–320 nm) and UVA (320–400 nm). However, at least 50% of the total energy that is being emitted by the sun and that reaches human skin is in the infrared (IR) range with wavelengths from 770 nm to 1 mm. Radiation of IRA range (770–1400 nm) represent one-third of the total solar energy and is well capable of penetrating human skin and directly affecting cells located in the epidermis, dermis, and subcutis.⁹ IRA radiation is strongly absorbed in mitochondria, where copper atoms present in complex IV of the respiratory chain might serve as the major chromophore.²³ However, there is no report of photosensitized production of $^1\text{O}_2$ with endogenous photosensitizers and radiation of the IRA so far.

In general, light absorption can be defined as a physical process that electronically excites a molecular system by nonionizing electromagnetic radiation such as photons. For an electronic transition of a molecule a usual wavelength range is the visible spectrum of light, ranging from 300–800 nm, in chemical energy of 150–400 kJ mol⁻¹. A photon with energy E (h : Planck's constant, ν : frequency of light)

$$E = h\nu \quad (11.1)$$

can be absorbed if that energy E is equal to the energy difference of the ground state (E_1) and an excited (E_2) in the photosensitizer molecule (λ : wavelength, c : speed of light)

$$E_1 - E_2 = \Delta E = h\nu = hc/\lambda. \quad (11.2)$$

The absorption of light in photosensitizer molecules leads to a decrease of an incoming light intensity I_0 to a value I , whereas the ratio of both is called

transmission T . The extent of light absorption is described by the following equation

$$T = I/I_0 = \exp(-\mu_a d), \quad (11.3)$$

where μ_a is the absorption coefficient of the photosensitizer and d is the thickness of the sample containing the photosensitizer. The value of μ_a strongly depends on the wavelength. Alternatively, the absorption of radiation A can be expressed as

$$A = 1 - T. \quad (11.4)$$

After absorption of radiation, the molecule can turn back from an excited state to the ground state by releasing the absorbed energy *via* internal conversion (heat) or fluorescence.

11.4. Endogenous Photosensitizers

If the radiation absorbing molecule is an endogenous photosensitizer, intersystem crossing from the excited singlet states to the triplet T_1 state may occur. The generation of $^1O_2$ requires energy of about 0.98 eV. Any excess energy of the absorbed radiation will dissipate within the molecule and to its neighborhood. Thus, any radiation energy that is absorbed in endogenous photosensitizers inside mammalian cells or tissue has the potential to yield $^1O_2$ with a certain quantum yield Φ_Δ (Figure 11.2).

Most of the known endogenous photosensitizers generate $^1O_2$ upon UVA irradiation (Table 11.1). However, many of these photosensitizers also absorb UVB radiation, sometimes to a higher extent as compared to UVA radiation (Figure 11.2). Thus, UVB-induced $^1O_2$ might play an additional, important role in the mechanisms of oxidative tissue damage. However, this issue has been rarely investigated in the past decades.^{24–26} Vitamin E (α -tocopherol) was found to generate $^1O_2$ under UVB-irradiation and its functional efficiency as antioxidant is now under discussion.²⁴ In view of the absorption spectra of flavins and protoporphyrin, visible radiation should also yield the generation of $^1O_2$.

The list of molecules that act as endogenous photosensitizers has been fairly extended during the past years and that list is still not exhaustive. It has been frequently mentioned that endogenous porphyrins and the flavins like riboflavin are ubiquitous and important photosensitizers.^{16,27} In fact, many porphyrins of the cellular heme synthesis like uroporphyrins, coproporphyrins and protoporphyrin IX (PPIX) are well known to be potent endogenous photosensitizers. Among these porphyrins, PPIX efficiently generate $^1O_2$ upon irradiation ($\Phi_\Delta = 0.56$).²⁸ This molecule is used for killing tumor cells in photodynamic tumor therapy (PDT).²⁹

Humans need different aqueous and liposoluble vitamins because of their important role as cofactors or coenzymes in human metabolism reactions.^{30,31} However, vitamins represent the major group of endogenous

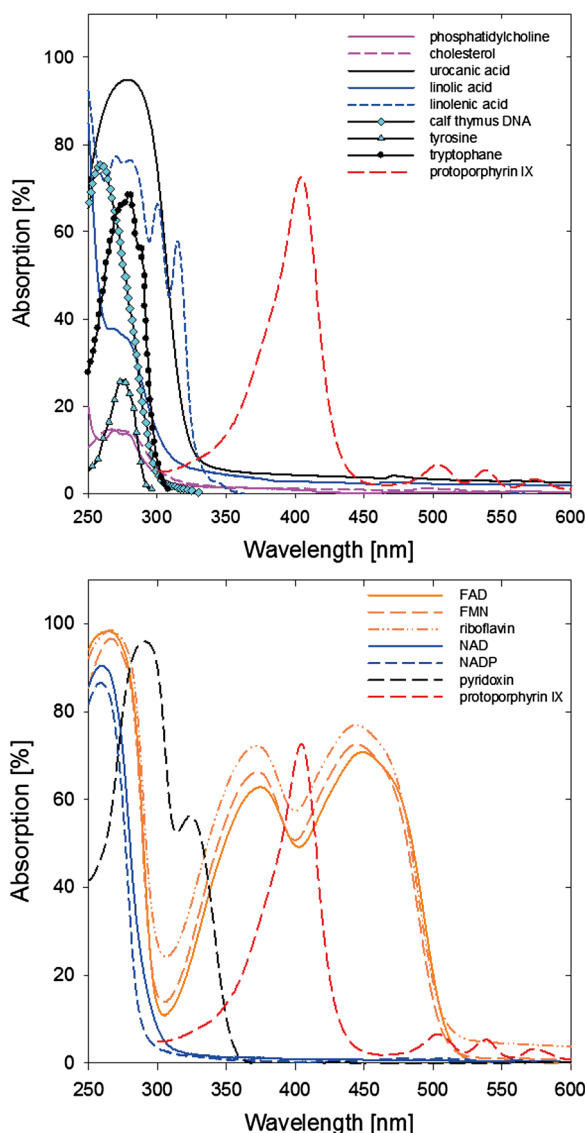


Figure 11.2. The absorption spectra are shown for endogenous photosensitizer such as nonvitamins (top) and vitamins (bottom). For better illustration, the absorption values are displayed in percent. The substances were dissolved in appropriate solvents at concentrations that allow its simultaneous presentation in the graph. For comparison, the nonvitamins graph (top) displays the absorption of DNA and exemplarily of two amino acids (tyrosine, tryptophane). The absorption values of protoporphyrin IX is shown in both graphs because this molecule, exemplarily for the other porphyrins of the heme synthesis, is assumed to be a major endogenous photosensitizer besides the flavins.

Table 11.1. Endogenous photosensitizers and quantum yield Φ_{Δ} .

Photosensitizer	Category	Φ_{Δ}	
		UVB (280–320 nm)	UVA (320–400 nm)
Protophyrin IX	Porphyrines	n.a. ^a	0.56 ²⁸
Vitamin A	Vitamin A	0.06 ³²	0.07 ³²
Riboflavin	Vitamin B ₂	0.61 ³²	0.54 ³⁷
			0.58 ³²
FMN	Vitamin B ₂	0.58 ³²	0.51 ³⁷
			0.58 ³²
FAD	Vitamin B ₂	0.13 ³²	0.07 ³⁷
			0.15 ³²
Nicotinic acid	Vitamin B ₃	0.05 ³²	— ^b
Nicotinic acid amide	Vitamin B ₃	0.64 ³²	— ^b
Pyridoxal 5'phosphate hydrate	Vitamin B ₆	0.16 ³²	0.13 ³²
Pyridoxal 5'phosphate	Vitamin B ₆	n.a.	0.56 ⁵⁷
Pyridoxal hydrochloride	Vitamin B ₆	0.06 ³²	0.06 ³²
Pyridoxine	Vitamin B ₆	0.11 ³²	0.08 ³²
Pyridoxamine	Vitamin B ₆	0.06 ³²	0.04 ³²
dihydrochloride			
Pyridoxyl- <i>p</i> -histidine	Vitamin B ₆	n.a.	0.17 ⁵⁷
Pyrocobester	Vitamin B ₁₂	n.a.	0.21 ⁵⁸
Ergosterol	Pro-vitamin D ₂	n.a.	0.85 ⁵⁹
Vitamin D ₂	Vitamin D ₂	0.06 ³²	— ^b
Vitamin D ₃	Vitamin D ₃	0.007 ³²	— ^b
Vitamin E	Vitamin E	0.15 ³²	— ^b
		0.17 ²⁴	
Vitamin K	Vitamin K	0.02 ³²	— ^b
Dihydropyridine (DHP)-lysine	MDA protein-epitope	n.a.	n.a. ⁴⁰
Lipofuscin	Aging pigment	n.a.	0.08 ⁶⁰
Pterin	UV receptors	n.a.	0.30 ⁴¹
Melanin	Endogenous pigment	n.a.	0.015–0.017 ^{6,8}

^aValue not available.^bNo absorption of radiation in the respective spectral range.^cExcitation at 532 nm, efficiency instead of quantum yield.

photosensitizers (Table 11.1). Excitation of riboflavin, FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) with UVA or UVB yielded strong luminescence signals and the respective quantum yields were determined with $\Phi_{\Delta} = 0.58$ –0.61 (riboflavin), $\Phi_{\Delta} = 0.58$ –0.64 (FMN), and $\Phi_{\Delta} = 0.13$ –0.15 (FAD).³² Depending on their concentration in cells, the flavins are potential generators of $^1\text{O}_2$, even more effective than exogenous porphyrins used for cell killing in photodynamic therapy. In view of these high values, it seems to be reasonable that these substances, even though at low concentrations, can provide sufficient amount of $^1\text{O}_2$ during radiation exposure that leads to gene regulation, photoaging, and possibly carcinogenesis.^{12,33} Also, other B vitamins exhibit the potential to generate $^1\text{O}_2$ upon UV irradiation and the quantum yields Φ_{Δ} ranged from 0.02 to 0.64 (Table 11.1).³²

One of the most important benefits claimed for vitamins A, C, E and many of the carotenoids is their role as antioxidants, which are scavengers of free

radicals, in particular when synergistic effects occur.³⁴ The vitamins A, D₂, D₃, and K are weak in generating ¹O₂, whereas vitamin E is a potent endogenous photosensitizer with Φ_{Δ} of 0.15.

Urocanic acid is a metabolite of histidine and a constituent of the horny layer making up 0.7% of the dry weight of the epidermis.³⁵ ¹O₂-initiated decomposition of urocanic acid was used to confirm indirectly that urocanic acid generates singlet oxygen when irradiated with UVA.³⁶ The generation of ¹O₂ upon UVA irradiation was directly proven by luminescence signals but without quantification.³⁷

Anhydroretinol is a metabolite of vitamin A (retinol) and a major photodecomposition product of retinyl palmitate and retinyl acetate. There is sufficient evidence that irradiation of anhydroretinol with UVA light generates reactive oxygen species, *e.g.* ¹O₂ that mediates the induction of lipid peroxidation.³⁸

The human retinal pigment epithelial (RPE) layer contains a complex mixture of components called lipofuscin. This mixture forms with age and with various genetic disorders such as Stargardt's disease. It is well accepted that lipofuscin generates ¹O₂ when excited with UVA, which contributes to retinal maculopathies.²⁷

The protein phosphatase calcineurin has been gradually revealed itself as the central controller of our immune response. UVA radiation suppresses calcineurin activity. Evidence was provided that this activity loss is partly due to ¹O₂ generated by photosensitization.³⁹ Experiments showed that the malondialdehyde-derived protein epitope dihydropyridine (DHP)-lysine is a potent endogenous UVA-photosensitizer of human skin cells.⁴⁰

Pterins (2-amino-4-hydroxypteridin derivatives) are a family of heterocyclic compounds present in a wide variety of biological systems. Pteroyl-L-glutamic acid (folic acid) is a precursor of coenzymes involved in the metabolism of nucleotides and amino acids. All investigated pterins significantly produced amounts of ¹O₂ with Φ_{Δ} in the range from 0.30 to 0.47 (pD value 10.5). Only folic acid showed a very small quantum yield of less than 0.02.⁴¹

Recent experiments showed that different species of melanin can generate ¹O₂ when irradiated with UVA or visible light.⁸ The authors utilized the term "efficiency of singlet oxygen" instead of "quantum yield" because melanin has a complex molecular structure and may be highly aggregated. That efficiency ranged from 0.015 to 0.017.

11.5. UV-Induced Generation of ¹O₂ – Atypical Endogenous Photosensitizers

Besides the photosensitized generation of ¹O₂, this reactive molecule can be produced by various chemical reactions involving different radicals and other reactive species.⁴² In the presence of oxidizable biomolecules like lipids, proteins or DNA, photosensitization and chemical reactions (*e.g.* lipid peroxidation) may occur at the same time yielding various products.^{43,44}

Usually, lipids and fatty acids are the target of $^1\text{O}_2$ that has been generated by any photosensitizer. $^1\text{O}_2$ -induced lipid peroxides subsequently trigger transcriptional activation of genes including the transcriptional activation of heme oxygenase 1.¹⁶ However, $^1\text{O}_2$ can be generated in suspensions of egg yolk phosphatidylcholine during irradiation with UVA that included the action of oxygen radicals.⁴⁵ Upon UVA irradiation, different fatty acids (oleic acid 18:1, linoleic acid 18:2, linolenic acid 18:3) in aerated ethanol solution (50 mmol L⁻¹ each) showed clear $^1\text{O}_2$ luminescence signals, without any photosensitizer. The decay time of the signal $\tau_{\Delta} = (K_{\Delta})^{-1}$ was in the range from 13 to 14 μs , which is the lifetime of $^1\text{O}_2$ in ethanol.^{46,47} However, the definition of the singlet oxygen quantum yield hampers the determination of Φ_{Δ} in that case.

Oxidized products of fatty acids must be present to enable initial absorption of UVA radiation.⁴⁸ Once singlet oxygen is generated, the amount of oxidized products increases, which in turn enhances radiation absorption. Usually, luminescence signals of photosensitizer-induced $^1\text{O}_2$ show a rise time and a decay time. However, time-resolved luminescence signals of $^1\text{O}_2$ in fatty acid solutions showed a decay time but no rise time. It is suggested that due to the lack of rise time, the light absorbing molecules are not able to form a triplet state (*e.g.* linear-shaped molecules like fatty acids). Thus, $^1\text{O}_2$ seems to be generated with the assistance of chemical reactions, but initiated by the applied UVA radiation.⁴⁹

Such fatty acids are major components of many cellular membranes that should underline their potential role in radiation mediated activation of cellular signaling. The skin contains a sufficient amount of oxygen ($p_{\text{O}_2} \sim 20$ Torr).⁵⁰ When exposed to UVA radiation, initial concentrations of oxidized fatty acids are present in skin^{51,52} to initiate the generation of $^1\text{O}_2$. In cells, a molecule such as ceramide is a key component of stress responses. UVA radiation and $^1\text{O}_2$ both generated ceramide in protein-free, sphingomyelin-containing liposomes.⁵³ Furthermore, human skin, especially the stratum corneum, contains free saturated and unsaturated fatty acids with mostly chain lengths of C₁₆ to C₁₈ atoms.⁵⁴

11.6. UV Radiation-Induced Changes of Endogenous Photosensitizers

Absorption of radiation by an endogenous photosensitizer such as vitamins and its ability to produce $^1\text{O}_2$ is very sensitive to the respective molecular structure. Any change of the molecular structure can immediately and substantially change absorption and $^1\text{O}_2$ production. Energy-rich UV radiation can change the structure of photosensitizer molecules depending on the wavelength and the applied energy. These changes, in particular during continuous, long-lasting irradiation, should affect the role of endogenous photosensitizers and its impact on cellular damage.

A recent investigation showed that UVB radiation can change the absorption spectra of vitamins in the entire absorption range of such molecules.⁵⁵

For nearly all vitamins investigated, a clear difference in absorption at least in the UVA or UVB region after UVB irradiation was detected. These data provide evidence that irradiation of vitamins with UVB can change the photo-physical features of these endogenous photosensitizers leading to a change of their potential of $^1\text{O}_2$ generation when exposed to UVB or UVA radiation. Thus, UVB photosensitizers like vitamin E also becomes an endogenous photosensitizer for UVA radiation after exposure to UVB.³²

This effect should play a major role in all experimental settings, in which UVA and UVB were consecutively applied to cells or tissue to detect any signaling or damaging effects.⁵⁶ These changes may even occur under parallel application of UVA and UVB radiation as for experiments with solar simulators or exposure to natural solar radiation. Such photochemical changes of endogenous photosensitizers along with the ability of $^1\text{O}_2$ generation may affect the interpretation of results regarding the role of UVA and UVB for skin damaging effects *in vivo*.

11.7. Conclusions and Outlook

UV-mediated cellular damage occurs in proteins and DNA, which are primary targets due to a combination of their UV-absorption characteristics and their abundance in cells. UV radiation can mediate damage *via* direct absorption of the incident light by the cellular components or photosensitization mechanisms, in which $^1\text{O}_2$ plays a major role. Many biomolecules, in particular vitamins, are potent endogenous photosensitizers yielding the specific DNA product 8-oxoG. The majority of UV-induced protein damage appears to be mediated by $^1\text{O}_2$, which reacts preferentially with certain side chains of amino acids. Such photo-oxidative reactions have an impact on pathological processes involved in the development of several disorders affecting radiation exposed tissues, the skin, the mucosa, and the eye.

Despite the extensive research of the past decades, it still necessitates further research to elucidate the role of $^1\text{O}_2$ in the cellular damaging mechanisms. The highly reactive $^1\text{O}_2$ can be generated by endogenous photosensitizers upon UV radiation (280–400 nm), but also visible light can contribute. In light of the different radiation wavelengths and the different cellular damages induced, excitation wavelength and radiant exposure should be correlated to the extent and the mechanisms of cellular damages.

In addition, little knowledge is available when applying UVA and UVB radiation to endogenous photosensitizers either alternating or in parallel. The latter application represents the natural solar radiation. Since UV radiation, in particular UVB, may change the chemical structure of endogenous photosensitizers, caution should be exercised when performing experiments with different UV sources at high radiant exposures. The capability of endogenous photosensitizers to produce $^1\text{O}_2$ may change in an unforeseeable way.

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Chapter 12

Endogenous Singlet Oxygen Photosensitizers in Plants[†]

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[†]This chapter is dedicated to the memory of María Cabeza Arellano (1964–2015), a dedicated and well-beloved teacher of biology.

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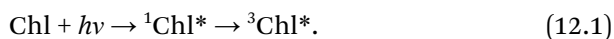
12.1. Introduction

Since all preliminary matters concerning the reactivity and photophysical properties of singlet oxygen ($^1\text{O}_2$) have already been dealt with in the preceding chapters, we need not dwell on these issues here, apart from reminding the reader that $^1\text{O}_2$ formation through photoexcitation of a ground-state oxygen molecule (O_2) has a negligibly small probability because the corresponding radiative transition is doubly forbidden, as it violates the selection rule for spin as well as that for electric-dipole radiation. This leaves photosensitization as the main feasible means of generating $^1\text{O}_2$. We proceed therefore to discuss the sensitization mechanisms through which $^1\text{O}_2$ is formed in plants. Additionally, we briefly introduce the formation and decomposition of some derivatives of lipid hydroperoxides as a possible source of chemical $^1\text{O}_2$ production in plants. The chapter also deals with photoprotection processes by which $^1\text{O}_2$ formation is prevented or $^1\text{O}_2$ is efficiently deactivated after its formation. Finally, the distance over which $^1\text{O}_2$ can diffuse in a viscous cellular medium, such as that found inside chloroplasts, is analyzed.

12.2. Mechanisms of Singlet Oxygen Production in Plants

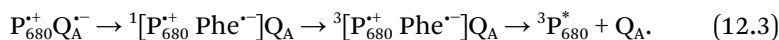
12.2.1. Photosensitization by Chlorophyll

A molecule with a singlet ground state can act as a photosensitizer (PS) when it is in its lowest triplet excited state ($^3\text{PS}^*$) or in its lowest singlet excited state ($^1\text{PS}^*$). The latter case will not be discussed here, because the singlet-state lifetime (τ_s^0) of the relevant PSs is too short, and the energy gap between $^1\text{PS}^*$ and $^3\text{PS}^*$ too small, to warrant contemplation in the present context. In order to be an efficient PS, a molecule must have a large molar absorption coefficient in at least one region of the visible spectrum, a large quantum yield for intersystem crossing (ISC) denoted as Φ_{IS} , and a long triplet lifetime, τ_{T}^0 . Among photosynthetic pigments, chlorophyll (Chl) molecules are by far the most important PSs in plants, although their derivatives, such as pheophytin (Phe), also meet all the above requirements. Photoexcitation of a Chl molecule to a high-lying level in the singlet manifold (Soret or Q_x band) is followed by a rapid (within a few ps or even faster) internal conversion to the lowest singlet excited state (Q_y , $^1\text{Chl}^*$), characterized by a lifetime (τ_s^0) of a few ns. In the absence of a fast competing event, which may be singlet excitation energy transfer (EET) to another Chl molecule or transfer of an electron to a neighboring molecule, the Q_y state of Chl makes a transition either to the ground state (through internal conversion or fluorescence emission), or to the first triplet excited state through ISC, leading to the formation of $^3\text{Chl}^*$ (eqn (12.1)).



In photosynthetic antenna complexes of plants, ISC is the only route for the formation of $^3\text{Chl}^*$, but in the reaction center (RC) of photosystem II (PSII),

$^3\text{Chl}^*$ may be formed not only by ISC but also by a process called the radical-pair mechanism.¹ Singlet EET between Chl molecules anchored in antenna complexes of PSII proceeds down an energy gradient until the excitation energy eventually reaches the primary electron donor of PSII RC, a Chl species denoted as P_{680} . At this point, charge separation takes place between the singlet excited state of P_{680} ($^1\text{P}_{680}^*$) and the primary Phe electron acceptor forming the singlet radical pair $^1[\text{P}_{680}^{+\bullet} \text{Phe}^{\bullet-}]$. If the electron transfer is blocked on the acceptor side of the RC and the electron cannot go beyond the primary plastoquinone electron acceptor (Q_A), $\text{P}_{680}^{+\bullet}\text{Q}_\text{A}^{\bullet-}$ charge recombination takes place and the electron returns to P_{680} . In this case, P_{680} can be formed after direct charge recombination (eqn (12.2)), when there are no intermediate species, or after indirect charge recombination (eqn (12.3)), when the triplet excited state of P_{680} ($^3\text{P}_{680}^*$) can be found as an intermediate species before the ground state P_{680} is newly repopulated. In the event of indirect charge recombination, $^1[\text{P}_{680}^{+\bullet} \text{Phe}^{\bullet-}]$ is formed and lasts for a few to several tens of ns depending on whether Q_A is reduced or absent. Since each radical ion is a spin doublet, and two doublets can form an overall singlet or triplet, the singlet radical pair $^1[\text{P}_{680}^{+\bullet} \text{Phe}^{\bullet-}]$ can be transformed, as a result of spin rephasing, into the triplet radical pair, $^3[\text{P}_{680}^{+\bullet} \text{Phe}^{\bullet-}]$, which subsequently recombines into $^3\text{P}_{680}^*$ and Phe.



The electronic transition from $^3\text{Chl}^*$ to the ground state Chl is spin forbidden and in the absence of triplet excitation energy acceptors, the τ_T^0 of $^3\text{Chl}^*$ is found in the range of 1.0 ms.² Here we find one of the conditions to produce high levels of $^1\text{O}_2$. The second circumstance, the value of Φ_T for $^3\text{Chl}^*$, is also propitious: it is found to be >0.6 for Chl molecules in solution³ and about 0.3 for $^3\text{P}_{680}^*$ in PSII RC complexes.⁴ All this is certainly an advantage if O_2 is the only acceptor of the excitation energy in the medium. The electronic energy transfer from $^3\text{Chl}^*$ to O_2 is almost diffusion controlled ($\sim 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) and $^1\text{O}_2$ production takes place after the formation of an encounter complex between $^3\text{Chl}^*$ and O_2 (eqn (12.4)).⁵

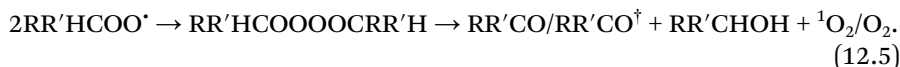


A list of the relative quantum yields of $^1\text{O}_2$ production (Φ_Δ) by Chl molecules and some of their derivatives in organic solvents and aqueous micellar systems using *meso*-tetraphenylporphyrin and tetra(*p*-sulfophenyl) porphyrin as standards is given by Krasnovsky.⁶ The value of Φ_Δ was determined to be ~ 0.16 in PSII RC.^{7,8}

12.2.2. Chemical Production by Lipid Hydroperoxides

The production of $^1\text{O}_2$ in plants is in most cases associated with the formation of $^3\text{Chl}^*$ in antenna complexes or $^3\text{P}_{680}^*$ in PSII RC, but there is no evidence for $^1\text{O}_2$ production in photosystem I (PSI).⁹ Nowadays, there are new

lines of evidence that support the view that $^1\text{O}_2$ can also be produced by other mechanisms in plants that do not require the formation of $^3\text{Chl}^*$ or $^3\text{P}_{680}^*$. The Russell mechanism is a process where $^1\text{O}_2$ is generated from lipid hydroperoxides (eqn (12.5)). In this case, the process starts with the combination of two peroxy radicals that form a linear tetraoxide intermediate, which undergoes a rapid decomposition, leading to the formation of several products including a ketone, an alcohol and O_2 . The reaction generates either a triplet excited ketone (denoted with a dagger) and $^3\text{O}_2$ or, in contrast, a ground state ketone and $^1\text{O}_2$.¹⁰



High light stress has been recognized to produce severe peroxidation of lipids in chloroplasts of *Chlamydomonas reinhardtii* and it has been suggested that $^1\text{O}_2$ detected in the cytoplasm of this algae is produced in the outer membrane of the chloroplast envelope by the Russell mechanism.¹¹ Recently, $^1\text{O}_2$ was also proposed to be formed in PSII under donor-side photoinhibition.¹² When the oxidized P_{680} ($\text{P}_{680}^{+\cdot}$) cannot be reduced by the redox active tyrosine Z (Tyr_Z), $\text{P}_{680}^{+\cdot}$ or $\text{Tyr}_Z^{+\cdot}$ can instead oxidize other molecules due to their high redox potentials and induce the formation of carbon-centered radicals that react with O_2 to form peroxy radicals.

12.3. Endogenous Singlet Oxygen Production by Photosynthetic Complexes of Plants

12.3.1. Antenna Complexes

The formation of $^1\text{O}_2$ in photosynthetic antenna complexes of plants is expected to take place if Chl molecules are not photoprotected by carotenoid (Car) molecules because the latter are either absent or are not in van der Waals contact with the former. When Car molecules are absent because their biosynthesis has been inhibited by chemical treatment with herbicides or a mutation has been introduced in the Car biosynthesis pathway, the effect can be so drastic sometimes that the photosynthetic organism undergoes a rapid photobleaching under aerobic conditions or exhibits a lethal phenotype. This is because the Car molecules perform, in addition to their photoprotective role, which entails quenching $^3\text{Chl}^*$, a structural role, which amounts to holding together the architectural ensemble of photosynthetic complexes, which becomes essential in some cases.^{13,14}

The quenching of $^3\text{Chl}^*$ by Car molecules is very efficient in antenna complexes of plants and consequently $^1\text{O}_2$ production is very low, provided that the antenna complexes are not damaged by light and the donor and acceptor pigments remain in van der Waals contact.¹⁵ In particular, the triplet EET from Chl *a* to xanthophyll (Xan) molecules in antenna complexes of PSII was established to be about 95%, leaving 5% of the $^3\text{Chl}^*$ unquenched.¹⁶

Mozzo and coworkers¹⁶ concluded that this incomplete quenching of $^3\text{Chl}^*$ is why other Car molecules, either free in the membrane or present at the interface between the lipids and proteins, are required to fully suppress $^1\text{O}_2$ formation. Interestingly, the different Xan molecules do not quench $^3\text{Chl}^*$ equally well while they are bound to the antenna complexes of PSII. The major light-harvesting complex (LHCII) of PSII, a pigment-protein complex consisting of three transmembrane α -helices, houses eight Chl *a* and six Chl *b* molecules and four Xan molecules [two lutein (Lut) molecules, one neoxanthin (Neo) and one Xan belonging to the Xan cycle, see below) and have a trimeric native structure (Figure 12.1).¹⁷ The Lut molecules occupying a central position in sites L1 and L2 of the antenna complexes of PSII, and particularly Lut in site L1 in close proximity to the Chl molecules responsible for the low-energy state of the complex, are the largest contributors to the quenching of $^3\text{Chl}^*$.¹⁶ In contrast to the Xan molecules occupying the L1 and L2 sites in the antenna complexes of PSII, the other two Xan molecules, Neo at the N1 site and violaxanthin (Vio) or zeaxanthin (Zea) at the V1 site, do not contribute directly in the quenching of $^3\text{Chl}^*$. The N1 site is very specific for Neo and it has been suggested that it is necessary for tightening the Chl-Chl and Chl-Car interactions in the antenna complex, which, in turn, favors efficient energy transfer between pigments and consequently a better quenching of $^3\text{Chl}^*$.¹⁸ The V1 site, which is at the periphery of the monomeric complex, can be occupied by Vio or Zea. In particular, when plants are exposed to high light, Vio is converted into Zea in the so-called Xan cycle to photoprotect PSII. In this process the excess light energy in the antenna complex of PSII is dissipated by nonphotochemical quenching (NPQ), that is, $^1\text{Chl}^*$ is deactivated and $^1\text{O}_2$ formation is prevented. The quenching capacity of Zea bound to the antenna complex is suggested to be enhanced by the distortion of its skeleton in the V1 site.¹⁹ In addition, Zea in the lipid membrane or bound to the antenna complex can directly quench $^1\text{O}_2$.

In spite of the presence of Xan molecules bound to the antenna complexes or free in the membranes, $^1\text{O}_2$ can be formed in thylakoids under photoinhibition. The yield of $^3\text{Chl}^*$ in thylakoids increases and the antenna complexes are more prone to photodamage, distinguishing the formation of $^3\text{Chl}^*$ in the antenna complexes from those produced in the PSII RC.²⁰ However, $^3\text{Chl}^*$ produced outside the RC do not correspond with free Chl molecules in the membrane, but with Chl molecules that are still bound to the damaged antenna complexes, where the Chl-to-Car distance undergoes subtle changes, which brings together uncoupled Chl molecules and consequently a large production of $^3\text{Chl}^*$.^{20,21} In particular, $^1\text{O}_2$ formation has been seen during the photoinhibition of isolated antenna complexes of PSII,²² where the Chl and Xan molecules are not in van der Waals contact in the photodamaged antenna complexes. When applying a more drastic effect on isolated PSII complex, the phosphorescence emission of $^1\text{O}_2$ was enhanced.²³ In this latter study, the PSII-enriched membranes were treated with sodium dodecyl sulfate and the Chl molecules were released from their specific binding site, bringing to an end any photoprotection by Car molecules.

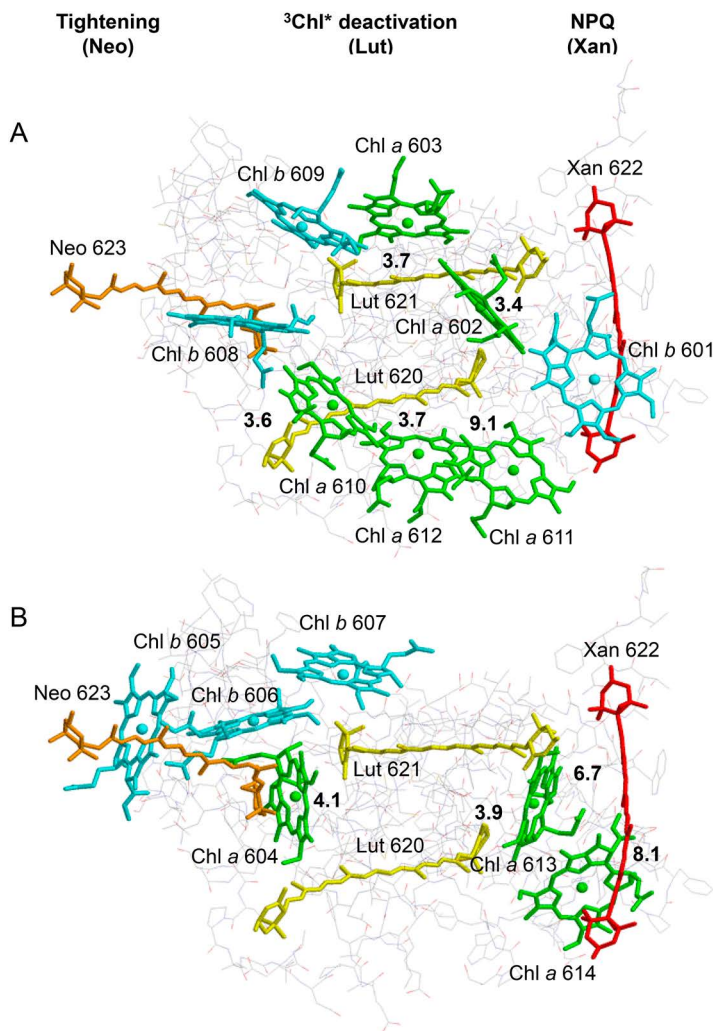


Figure 12.1. Pigment arrangement in LHCII of plants on the stromal (A) and lumenal (B) sides, respectively. View parallel to the membrane normal from the stromal side. The photoprotection role of Car molecules to prevent $^1\text{O}_2$ formation by several means is given: NPQ role for Xan (*i.e.* zeaxanthin) occupying the V1 site, Xan 622; $^3\text{Chl}^*$ deactivation role for Lut molecules occupying the L1 and L2 sites, Lut 620 and Lut 621; and a tightening role for Neo occupying the N1 site, Neo 623. Relevant closest distances between the π - π systems of Chl *a* and Car molecules are in bold and given in angstroms. For the sake of clarity, the phytol chains of Chl *a* and Chl *b* molecules have been truncated. The numbers ascribed to the pigment molecules correspond with those given in the PDB file with accession code 1RWT (<http://www.pdb.org>).

12.3.2. Photosystem II Reaction Center (PSII RC)

PSII RC consists of two polypeptides subunits denoted D1 and D2, the cytochrome b_{559} subunits PsbE and PsbF, and the small subunit named PsbI.^{24,25} The protein complex of PSII RC in the most stable and active form houses six Chl a molecules, two Phe a molecules and two β -Car molecules in the heterodimer D1/D2. The two β -Car molecules are in the *trans* configuration, one in the D1 protein (β -Car_{D1}) close to a peripheral Chl denoted Chl_{zD1} with nearly a perpendicular orientation with regard to the membrane plane and another in the D2 protein (β -Car_{D2}) close to another peripheral Chl denoted as Chl_{zD2} with a parallel orientation with regard to the membrane plane. After indirect charge recombination, the triplet excitation energy is mainly localized in the accessory Chl of the D1 protein (Chl_{D1}) and only a minor population in the primary donor P₆₈₀, consisting of two Chl molecules denoted P_{D1} and P_{D2} located at the interface of the D1 and D2 proteins close to the luminal side.²⁶ As a consequence of the pigment arrangement in the PSII RC complex dictated by the protein matrix,²⁷ the distance of the two β -Car molecules to the Chl_{D1} and P₆₈₀ is well beyond van der Waals contact (Figure 12.2(A)) and so triplet EET is very inefficient from $^3P^*$ to the β -Car molecules, where $^3P^*$ represents

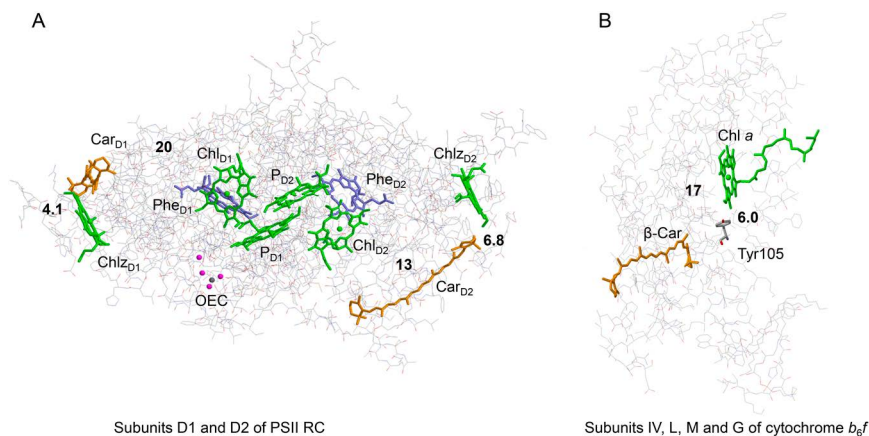


Figure 12.2. Pigment arrangement in PSII RC (A) and cytochrome b_6f (B). The primary electron donor P₆₈₀ in PSII RC is unprotected by β -Car molecules. The distance of the two β -Car molecules to the Chl_{D1} and P₆₈₀ is well beyond van der Waals contact and so $^1O_2$ can be formed by triplet EET from $^3P^*$. View parallel to the membrane normal from the luminal side. For the sake of clarity, the phytol chains of Chl a molecules have been truncated. The definition of the abbreviations for the pigments is given in the main text. Atoms in magenta and gray represents the Mn₄Ca cluster of the oxygen evolving complex (OEC) of PSII. The Chl a in the subunit IV of the cytochrome b_6f is proposed to form a radical pair with Tyr150 of the cytochrome b_6 shortening the τ_s of $^1Chl^*$. View parallel to the membrane plane. Relevant closest distances between the π - π systems of Chl a and β -Car molecules and edge-to-edge between Chl a and Tyr 150 in PSII RC and cytochrome b_6f are in bold and given in angstroms. PSII RC, PDB file with accession code 2AXT, and cytochrome b_6f , PDB file with accession code 1VF5 (<http://www.pdb.org>).

the total population of $^3\text{Chl}^*$ in the PSII RC (*i.e.*, $^3\text{Chl}_{\text{D1}}^*$ and $^3\text{P}_{680}^*$ in thermal equilibrium). The actual triplet-state lifetime (τ_{T}) of $^3\text{P}^*$ was determined to be 600–1000 μs under anaerobic conditions, 20–40 μs under aerobic conditions and 4–6 μs under oxygenic conditions,^{28,29} which clearly indicates that $^3\text{P}^*$ can be efficiently quenched by O_2 , even in the presence of $\beta\text{-Car}$ in PSII RC. Under anaerobic conditions and in the absence of electron acceptors, the triplet-minus-singlet spectrum of PSII RC complexes shows the recognized features of $^3\text{P}^*$ as a result of the indirect charge recombination (Figure 12.3(A)). This signal is accompanied with short delays by a small contribution that corresponds with the formation of $^3\beta\text{-Car}$ on account of its spectral features and rapid decay.^{1,30} Of the two $\beta\text{-Car}$ molecules in the PSII RC, only the one within the D1 protein is in van der Waals contact with the peripheral Chl_{D1} , implying that the triplet EET from Chl_{D1} to the adjacent $\beta\text{-Car}$ must be responsible for the observed short-lived spectral feature. The absence of an efficient mechanism that prevents the formation of $^1\text{O}_2$ in the PSII RC has a rapidly detrimental effect on the pigment and proteins of this complex.^{4,31,32} The most obvious damage was the photobleaching of P_{680} that (surprisingly) was accompanied with loss of $\beta\text{-Car}$. Additionally, the photodamage to the D1 (and D2) proteins could be determined by Western blot analyses on the basis of the changes in the intensity and electrophoretic mobility of the protein bands.^{31,33}

The PSII RC from higher plants was the biological system where direct emission at 1270 nm from $^1\text{O}_2$ with an endogenous origin was first observed.⁷ In the study by Macpherson and coworkers,⁷ $^1\text{O}_2$ could be detected both when the primary charge separation was active and when it was inactive, suggesting that in the first case $^1\text{O}_2$ generation depended on the formation of $^3\text{P}^*$ by the radical-pair mechanism and in the second case on the formation of uncoupled $^3\text{Chl}^*$ by ISC. The Φ_{Δ} was determined to be about 0.16, about half of the quantum yield of $^3\text{P}^*$, suggesting that a pool of $^1\text{O}_2$ formed in the interior of the PSII RC was quenched rapidly by the pigments and protein matrix before diffusing out into the surrounding medium.⁸ The addition of physical or chemical quenchers (Qs), such as sodium azide, histidine or imidazole, or the replacement of deuterium oxide with water did not protect the pigments and proteins of the PSII RC from photodamage,^{7,34} although a partial protection of the surface-exposed regions of the protein matrix of the D1 and D2 polypeptides was observed when the water-soluble analog of $\alpha\text{-tocopherol}$, *i.e.* Trolox, was attached to the detergent-embedded preparations of PSII RC.³¹ In the 1990s the temporal profile of the phosphorescence emission of $^1\text{O}_2$ endogenously produced by PSII RC was technically possible using Ge or InGaAs detectors after the replacement of water with deuterium oxide. With the advent of NIR-sensitive photomultiplier tubes, efforts to measure the temporal profile of the phosphorescence emission in PSII RC were undertaken in aqueous buffer.³⁵ In this former study, a weak signal was discernible whose kinetic traces were well fitted to a biexponential function. Intriguingly, the larger rate constant ($3\text{ }\mu\text{s}$)⁻¹ was found to be independent of whether the experiment was performed under aerobic or oxygen-saturated conditions; whereas, the smaller rate constant varied from ($\sim 12\text{ }\mu\text{s}$)⁻¹ under aerobic

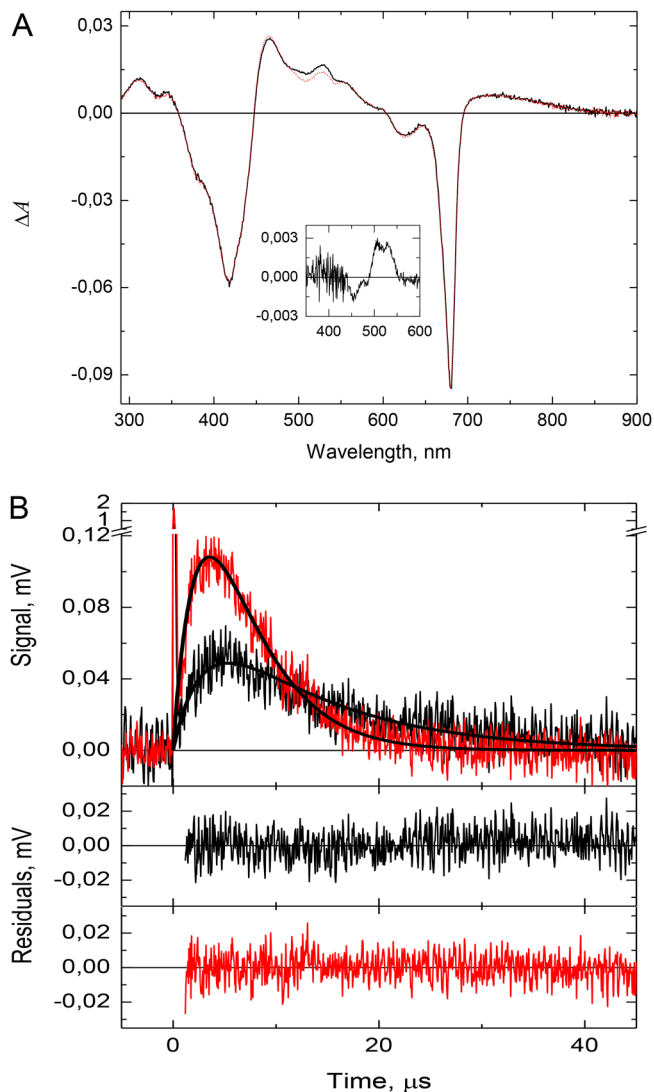


Figure 12.3. (A) Room-temperature photoinduced absorption difference spectra of the five-chlorophyll PSII RC under anaerobic conditions at delays of 5 μ s (solid line in black) and 30 μ s (dotted line in red); the two difference spectra are normalized to the same amplitude at 680 nm. Each curve is an average of 120 spectra. Inset, subtraction of the normalized 5 μ s and 30 μ s difference spectra in the spectral region where the Car singlet ground state and $^3\text{Car}^*$ absorb. Reproduced from ref. 30 with permission from the American Chemical Society. (B) Temporal profile of the emission signal at 1270 nm of $^1\text{O}_2$ produced endogenously by of the five-chlorophyll PSII RC in 20 mM Tris-HCl pH 7.2 under aerobic (black line) and oxygenic (red line) conditions. The absorbance of the five-chlorophyll PSII RC (for a path length of 1 cm) is 1.5 at 675 nm. The discontinuity in the y-axis illustrates the intensity of additional radiation reaching the NIR-sensitive photomultiplier tube. The number of averaged scans is 6144 and 5120 for aerobic and oxygenic conditions, respectively. The best fit for each signal and the residuals are also shown. Reproduced from ref. 35 with kind permission from Springer Science and Business Media.

conditions to $(\sim 4 \mu\text{s})^{-1}$ under oxygen-saturated conditions, suggesting that the oxygen-independent rate constant corresponded with the rate constant for the actual $^1\text{O}_2$ decay (k_Δ) and the oxygen-dependent rate constants were in fact the rate constants for the $^1\text{O}_2$ formation ($k_{f,\Delta}$) (Figure 12.3(B)). Both formation rate constants, $k_{f,\Delta}$, went in parallel with the changes in the rate constant for the actual triplet-state decay (k_T) of $^3\text{P}^*$ under aerobic and oxygen-saturated conditions.⁸ Extrapolations based on the low amplitude of the phosphorescence emission of $^1\text{O}_2$ formed by PSII RC in aqueous buffers, together with the expected values of the rate constants for the formation and deactivation of $^1\text{O}_2$ in chloroplasts (see below), indicate that the experimental determination of $k_{f,\Delta}$ and k_Δ of the phosphorescence emission of $^1\text{O}_2$ endogenously produced by PSII in chloroplasts would be a tall order indeed.³⁵

12.3.3. Cytochrome b_6f

The integral membrane cytochrome b_6f complex plays a crucial role in electron and proton transfer in oxygenic photosynthesis. It mediates electron transport between PSII and PSI by oxidizing plastoquinol and reducing plastocyanin (or cytochrome c_6), while enabling coupled proton translocation across the thylakoid membrane.³⁶ The crystal structure of cytochrome b_6f clearly shows that the subunit IV of the cytochrome b_6f binds one Chl a molecule and that one Car molecule lies near the center of the transmembrane region between the PetL and PetM subunits. Intriguingly, the closest distance between both molecules is about 14 Å, too large for efficient triplet EET from Chl to Car (Figure 12.2(B)).^{37,38} This implies that the Car molecule exerts a poor photoprotection role in the cytochrome b_6f . The formation of $^3\text{Chl}^*$ has been observed in isolated cytochrome b_6f complexes, but the Φ_T and τ_T of $^3\text{Chl}^*$ was found to depend on the oligomeric state of the complex.³⁹ When it was solubilized with n -dodecyl- β -D-maltoside, cytochrome b_6f maintained its native assembly and $^1\text{O}_2$ production was low under aerobic conditions. In contrast, $^1\text{O}_2$ production was enhanced when its structural integrity was lost with n -octyl- β -D-glucopyranoside or sodium dodecyl sulfate. No evidence for triplet EET to the Car molecule or amino acid residues was found. This allowed Ma and coworkers³⁹ to conclude that the integrity of the cytochrome b_6f was crucial to prevent the formation of $^3\text{Chl}^*$ and further photosensitization of $^1\text{O}_2$. Alternatively, Dashdorj and coworkers⁴⁰ proposed an unconventional photoprotection mechanism where charge transfer between $^1\text{Chl}^* a$ and a nearby aromatic residue was invoked to be responsible for the shortening of the τ_s of $^1\text{Chl}^*$ (eqn (12.6)).



12.3.4. Chlorophyll Derivatives and Free Chlorophyll Molecules

In the above subsections, $^1\text{O}_2$ production has been described in mature photosynthetic complexes. However, the biogenesis and degradation of photosynthetic complexes or stress conditions bring to the stage precursors or

degradation products of Chl molecules, which in some cases are characterized by being very active in photosensitizing $^1\text{O}_2$. These PSs may accumulate at high concentrations in some mutants with defects in the biosynthesis or degradation pathways of Chl molecules, when inhibitors blocking the metabolic routes of Chl are present or under high light irradiance causing the release of Chl molecules from their binding sites. The addition of fosmidomycin has been recently demonstrated to inhibit the methylerythritol phosphate biosynthesis pathway that is required for the production of isoprenoid lipids.⁴¹ The result is a stoichiometric imbalance between Chl precursors and isoprenoid lipids that produces the accumulation of chlorophyllide and, consequently, the light-dependent death of the plant due to $^1\text{O}_2$ photosensitization. The *flu* mutant of *Arabidopsis* contains a mutation in a negative regulator of Chl biosynthesis that results in enhanced production of protochlorophyllide (Pchlde), which also produces a surge in $^1\text{O}_2$ production after a dark-to-light shift.⁴² Another Chl precursor known to produce $^1\text{O}_2$ is protoporphyrin IX.⁴³ It accumulates in plants treated with photobleaching herbicides such as acifluorfen and oxadiazon.⁴⁴ When *Arabidopsis* plants are challenged with *Pseudomonas syringae* pv *tomato*, the *staygreen* transcript encoding a protein, which promotes Chl degradation through the disruption of antenna complexes, is upregulated. The Chl catabolite pheophorbide is detected a few hours later, together with an increase in $^1\text{O}_2$ production and the activation of a hypersensitive response in *Arabidopsis*.⁴⁵ The accumulation of other Chl catabolites as the red Chl catabolite in a mutant of *Arabidopsis* with defects in the red Chl catabolite reductase is manifested by light-dependent death phenotype, which is also attributed to $^1\text{O}_2$ production.⁴⁶

Under normal physiological conditions, Chl intermediates are not free in the medium. They are bound to their respective enzymes or carriers close to the site of insertion into the photosynthetic complex during biogenesis⁴⁷ or the site from delivery during senescence.⁴⁵ This ensures that the detrimental effect they can provoke is minimal. The early light-induced proteins (ELIP) are known to be involved in the binding of Chl molecules released during the proteolytic degradation of LHCII^{48,49} or the D1 protein of PSII RC.⁵⁰ When the Chl molecules is bound to ELIP, it is proposed that $^3\text{Chl}^*$ is deactivated by triplet EET to a Xan molecule also bound to ELIP.⁵¹ The water-soluble Chl protein (WSCP) is a carrier of Chl, but also of Chl precursors and even Chl degradation products that still bind the central Mg ion.⁵² However, WSCP does not bind Xan molecules in contrast to ELIP. Nevertheless, the Φ_{Δ} of Chl bound to WSCP is about four times lower than that of free Chl, which suggests that a photoprotection mechanism must be present in this protein. Among some plausible explanations, it has been proposed that the Chl molecule being tightly packed by the WSCP tetramer avoids the encounter with O_2 . Overall, the accumulation of Chl precursors or catabolites is responsible for cell photodamage and strict control of their metabolic pathways is thus required to adjust their production, including biosynthesis regulation,⁵³ redox control⁵⁴ or transcriptional regulation.⁴⁵

12.4. Prevention of Singlet Oxygen Formation in Plants

12.4.1. Triplet Excitation Energy Transfer from Chlorophyll Molecules to Carotenoid Molecules

Triplet EET is a process that needs proper matching between the energy levels of the transfer partners, the donor and the acceptor, and their contiguity, since the transfer is mediated by electron exchange, an extremely short-range interaction. This is a process that can be observed after the encounter of the donor and acceptor in solution, where there are no structural restrictions. Consequently, high concentrations of the acceptor are necessary for the transfer to be an efficient process in solution. However, Car and Chl molecules are usually found in a balanced stoichiometric ratio in photosynthetic complexes of plants, indicating that the photosynthetic complex must provide a close contact between Chl and Car molecules to ensure efficient triplet EET to the donor molecule and also to avoid $^1\text{O}_2$ formation. Indeed, calculations on triplet EET between Chl and Car molecules in antenna complexes of photosynthetic organisms show how the rates depend on the degree of the π - π contact of the full π delocalization area of the donor and acceptor molecules and how such electronic coupling permits energy transfer rates in the nanosecond or picosecond time scale.⁵⁵ You and coworkers⁵⁵ concluded that the triplet-state electron density was almost equally distributed along the full π delocalization area and was not perturbed by molecular substituents, which is advantageous for optimizing the wavefunction overlap between the donor and acceptor molecules. In another study, Di Valentin and coworkers⁵⁶ investigated the electronic and structural requisites for triplet EET in the peridinin-Chl *a* protein of dinoflagellates and in LHCII; they emphasized the occurrence of Chl-Car pairs in van der Waal contact *and* the presence, at the interface, of a bridging molecule (in the one case a water molecule, and in the other an amino acid) which plays a crucial role in mediating a more favorable triplet EET between the pigments.

Together with the interaction requirements, the Chl-to-Car triplet EET also depends on the triplet-state energy (E_T) of Chl and Car. There are very few studies where the triplet excitation energy of Car molecules has been experimentally determined and most of them are restricted to Car molecules with a small number of conjugated double bonds (N). The experimental determination of the E_T of Car molecules with $N > 6$ is very difficult based on the fact that $^3\text{Car}^*$ mainly decays *via* nonradiative conversion and the phosphorescence emission is extremely weak. Because of this, the E_T of Car molecules with $N > 6$ has been assigned a value by extrapolation from those with $N < 6$ or has been proposed to be approximately half of that of the lowest singlet excited (S_1) state of Car molecules.⁵⁷ Efforts to measure the phosphorescence emission of β -Car have been carried out in solution for β -Car,⁵⁸ although the value reported was questioned later. Precise values for the E_T of Car with $N > 6$ are not known, but experiments with Car and

Chl molecules in solution or with Car-reconstituted and natural photosynthetic complexes show that the E_T of Car with $N \geq 9$ is below both the E_T and E_A of Chl and $^1O_2$.⁵⁹ So, in the first place, Car molecules prevent Chl molecules from $^1O_2$ photodamage by accepting the triplet excitation energy of Chl and, in the second place, they can deactivate $^1O_2$ and therefore photoprotect Chl molecules, if eventually $^1O_2$ is formed after molecular collision with $^3Chl^*$.

12.4.2. Nonphotochemical Quenching

NPQ is a complex photoprotection mechanism developed to avoid the photodamage of the photosynthetic apparatus by reactive oxygen species (ROS) and particularly $^1O_2$. In this photoprotection mechanism the PSII switches from a light-harvesting state to an energy-dissipation state, where the excess of the absorbed light energy is dissipated harmlessly as heat.^{60,61} At this point it is important to stress that NPQ is a photoprotection mechanism where $^1Chl^*$, but not $^3Chl^*$, is quenched. Several components contribute to NPQ. The major one is denoted qE and it depends on the pH of the thylakoid lumen, the activation of the PsbS, a pH sensing protein, and the rapid interconversion of epoxidized Xan molecules (Vio) into de-epoxidized Xan molecules (Zea *via* antheraxanthin) through the enzymatic Xan cycle. The state transition component (qT) involves the migration of LHCII from PSII to PSI after reversible phosphorylation in order to balance the absorbing photons between both photosystems. Nonetheless, its participation is small in comparison to the qE component and other NPQ components. The component qZ has been identified as a slowly developing and slowly relaxing component of NPQ that also depends on the formation of Zea, but, in contrast to qE, it is pH independent.⁶² The last component qI is a very slow relaxing component and it is associated with the sum or contribution of several processes that induce a severe inactivation of PSII; some of them related to the turnover of the D1 protein of the PSII RC and the maintenance of a proton gradient across the thylakoid membranes. A Lut cycle, where Lut-5,6-epoxide is converted into Lut in the Neo site of LHCII of some plant species, has also been shown to contribute to NPQ quenching;⁶³ although the main photoprotection role of Lut in the antenna complexes of PSII is restricted to the deactivation of $^3Chl^*$. The Xan interconversion is responsible for conformational changes or changes in the protein-protein interactions in the antenna complexes that avoid the excess of light energy to reach the PSII RC and, consequently, the formation of $^1O_2$ and other ROS. After the formation of Zea, $^1Chl^*$ is deactivated. However, the invoked mechanism shows some differences between LHCII and the minor antenna complexes of PSII. In LHCII, it is claimed that LHCII aggregation induces changes in the interaction between Chl molecules that bring together the formation of a Chl-Chl charge-transfer state.^{64,65} This charge-transfer state within the Chl homodimer is suggested to have an enhanced coupling to the ground state that causes a rapid direct recombination to the ground state.

However, there is no evidence for singlet EET from the $^1\text{Chl}^*$ molecules to the S_1 state of the Xan molecules or for the formation Xan cations in LHCII. In contrast to LHCII, a charge-transfer state is claimed to be formed between Chl and Zea molecules in the minor antenna complexes of higher plants and also to be responsible for the deactivation of the light energy funnelled to the RC.⁶⁶ The yield of charge-transfer state within the Chl-Zea heterodimer was much higher in thylakoids than in isolated minor antenna complexes, suggesting that protein interactions and changes in the lumen pH were required. The NPQ process in LHCII requires the activation of the PsbS and a change to low pH in the lumen (*i.e.* qE), whereas a PsbS-independent NPQ has been described in the minor antenna complexes of PSII.⁶⁷ This still leaves unanswered the question of which component of the NPQ, qE or qZ, contributes most in the minor antenna complexes of PSII. In summary, the formation of Zea contributes to both the NPQ photoprotection mechanism by deactivation of $^1\text{Chl}^*$ in the major and minor antenna complexes of PSII (qE and qZ) and the quenching of $^1\text{O}_2$ when Zea is free in the thylakoids, avoiding the photodamage of the antenna complexes and PSII RC (qI).⁶¹

12.4.3. Changes in Redox Potential of Plastoquinone A

The modification of the reduction potential (E_{red}) of Q_A of PSII RC is observed during the assembly of PSII or under stress conditions.^{68–70} As a consequence of the E_{red} shift of Q_A , the direct (or indirect) pathway of the charge recombination $P_{680}^{+*}Q_A^{-*}$ can be enhanced or impeded, observing an increase in $^1\text{O}_2$ production when the indirect recombination is favored. Q_A can be present in two different redox potential forms: a low potential form that is observed when the PSII is active and a high potential form that is present when PSII is inactive. The shift between these two redox potential forms has a key physiological role in the photoprotection of PSII. When the Mn cluster is not still bound to the donor side of PSII during the PSII assembly, the high redox potential form of Q_A is found⁶⁹ and the electron transport from Q_A to the secondary plastoquinone electron acceptor Q_B is not energetically favorable. In this case, direct charge recombination of $P_{680}^{+*}Q_A^{-*}$ to the ground state of P_{680} occurs without $^1\text{O}_2$ production. After the photoactivation of PSII, the E_{red} is switched to the low potential form and PSII becomes functional. A reverse switch of the E_{red} of Q_A can also be observed. Under high-light conditions, the pH of the lumen decreases and can reach a value low enough to induce the release of the Ca ion from the donor side of PSII.^{71,72} The loss of Ca affects the Q_A -binding site on the acceptor side, most probably through conformational changes, shifting the E_{red} of Q_A to high values. Attempts to monitor $^1\text{O}_2$ production in preparations of PSII where Ca and Mn ions were removed did not succeed, confirming that direct charge recombination was enhanced.⁷³ Changes in the E_{red} of both Q_A and Q_B have also been observed to confer resistance to photoinhibition in low-temperature-acclimated plants of *Arabidopsis*.⁷⁴ In this case, a downshift of E_{red} of Q_B accompanied with an upshift of the E_{red} of Q_A was

proposed to favor direct charge recombination, decreasing the Φ_T of ${}^3P_{680}^*$, and consequently ${}^1O_2$ formation. Likewise, changes in the E_{red} of Phe in cyanobacterial PSII RC were also suggested to be an acclimation response of cyanobacteria to changing environmental conditions.⁷⁵ The D1 and D2 proteins of the PSII RC from cyanobacteria are encoded by a small multifamily of genes, whose expression and distribution respond to different stress conditions. Under high-light conditions, the Gln130Glu, Leu151Ser and Ser124Phe replacements in the D1 protein enhance the yield of the nonradiative charge recombination of the radical of $P_{680}^+Q_A^-$ to the ground state of P_{680} , diminishing ${}^1O_2$ production. Furthermore, the inhibition of the acceptor side by herbicides that bind the Q_B site has been reported to modify the E_{red} of Q_A to higher and lower values, depending on the nature of the herbicide.⁷⁰ The phenolic herbicide bromoxynil downshifted the E_{red} of Q_A , but the urea-type herbicide DCMU upshifted it. Electron paramagnetic resonance analysis revealed that ${}^1O_2$ production with bromoxynil was twice that with DCMU.⁷⁶ Direct mutagenesis of amino acid residues in the vicinity of Q_A , Phe and P_{680} have also been reported to shift the E_{red} of these electron carriers in the PSII RC, and consequently the Φ_A .^{77–80}

12.5. Deactivation of Singlet Oxygen

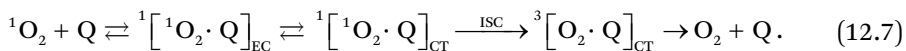
12.5.1. Physical Deactivation

In the absence of chemical quenching of ${}^1O_2$ by biological compounds, three main radiationless deactivation processes prevail over the radiative process characterized by phosphorescence emission at 1270 nm. These three processes include electronic energy transfer, charge transfer and electronic-vibrational energy transfer.⁸¹ Undoubtedly, the most efficient radiationless deactivation in photosynthetic organisms is the electronic energy transfer between ${}^1O_2$ and Car molecules. Foote and Denny⁸² first demonstrated physical quenching of ${}^1O_2$ by β -Car. They proposed a mechanism where electronic energy transfer between both compounds would be involved, adding that if this were the case the E_T of β -Car had to be near to or below that of ${}^1O_2$. The electronic energy transfer process was later demonstrated by Farmilo and Wilkinson,⁸³ when they observed the formation of ${}^3\beta\text{-Car}^*$. Since then, there has been intense research into electronic energy transfer from ${}^1O_2$ and Car molecules. The main results indicate that the ability of Car molecules to quench ${}^1O_2$ —when they are free in solution—depends on N , failing to quench efficiently ${}^1O_2$ if $N < 8$.^{84,85} The bimolecular rate constant (k_A^Q) for the electronic energy transfer approaches the diffusion-controlled rate ($10^{10} \text{ M}^{-1} \text{ s}^{-1}$) in solution; however, Truscott and coworkers⁸⁶ observed that the k_A^Q for the deactivation of ${}^1O_2$ by β -Car and Lyc barely decreased upon a threefold increase in the viscosity of the solvent. Schmidt^{5,87} showed that the changes in the k_A^Q for the quenching of ${}^1O_2^*$ by Car could be well fitted to the excess energy between the (estimated) E_T of Car molecules and the E_A of ${}^1O_2^*$.

However, when physical quenching is investigated in model membranes, the results are markedly different and the k_{Δ}^Q does not only depend on N , but on their structure, mobility and ability to form aggregates in the membranes.⁸⁸ To the best of our knowledge, the k_{Δ}^Q for the deactivation of $^1O_2$ by free Car molecules in thylakoids has not been studied and the expected behavior can be extrapolated for studies in model membranes. The k_{Δ}^Q for β -Car, Zea, Lyc and Lut were 1–2 orders of magnitude lower in liposomes than in organic solvents.⁸⁸ Both β -Car and Lyc are randomly dispersed in model membranes and increase membrane fluidity; of all the Car molecules with $N = 11$, these two exhibited the largest k_{Δ}^Q , and the quenching of $^1O_2$ did not depend on whether it was generated in the liquid or lipid phase. The mobility of Zea in membranes is hampered by the interaction of its hydroxyl group with the polar head of lipids, inducing a rigidifying effect that obstructs O_2 diffusion. The ability of Zea to quench $^1O_2$ decreases at high concentrations, when it forms aggregates in model membranes. Lut with $N = 10$ exhibited the lowest k_{Δ}^Q and also tended to form aggregates. The ability of Car molecules to quench $^1O_2$ in carotenoproteins has also been investigated, but the available information is scarce in comparison with studies in model membranes. Kawasaki and coworkers⁸⁹ isolated an aqueous carotenoprotein containing astaxanthin in an eukaryotic alga whose expression was induced under photo-oxidative conditions. This carotenoprotein was demonstrated to quench $^1O_2$; however, on the basis of its location in the periplasmic space of the cell it was proposed that it could not protect the cell from $^1O_2$ produced by the PSII, but by other PSs that might be present in this cellular space.

In most photosynthetic complexes, Car molecules are in van der Waals contact with Chl molecules, ensuring an efficient energy transfer between them. However, there are photosynthetic complexes such as PSII RC or cytochrome b_6f where the distance between Car and Chl molecules are well beyond van der Waals contact. Or there are stress conditions that damage the photosynthetic complexes and bring about subtle changes in the interaction between Chl and Car molecules and a concomitant release of free Chl molecules or Chl derivatives. Under these circumstances $^1O_2$ photosensitization by these hazardous PSs is enhanced and $^1O_2$ can randomly photodamage them or other biological compounds that are close to the Chl binding sites or are simply in the vicinity. It is then when lipophilic and hydrophilic antioxidants (vitamin E, C, B_6 or sulfide compounds, *etc.*) come to the fore to take part in the deactivation of $^1O_2$ by charge-transfer deactivation. This second deactivation process also requires the encounter of $^1O_2$ with Q, but the k_{Δ}^Q is up 1–7 orders of magnitude lower than that of electronic energy transfer. This is counterbalanced by relatively high concentrations (10^{-3} – 10^{-1} M) of Qs in intracellular media.^{90,91} In this case, the Qs cannot accept the E_{Δ} of $^1O_2$ by energy transfer because their E_T is higher. Instead, the ability of Qs to deactivate $^1O_2$ by charge transfer depends on their oxidation potential (E_{ox}). Both Q and $^1O_2$ form an encounter complex of singlet multiplicity that is stabilized by charge transfer from the former to the latter. This exciplex undergoes ISC

and decays to a triplet ground state that eventually dissociates to O_2 and Q (eqn (12.7)).⁸¹



The charge-transfer deactivation is promoted by the high electron affinity of $^1O_2$ and, consequently, the k_{Δ}^Q increases when the E_{ox} of Q decreases. The rate constant follows an empirical correlation with the free-energy change of the complete electron-transfer process established by Rehm and Weller.⁹² An inverse linear correlation between the logarithmic function of the overall k_{Δ}^Q and the E_{ox} of Q was demonstrated for Chl molecules, Chl derivatives and phenolic compounds of biological interest.^{93–95} Similarly, when the k_{Δ}^Q of vitamin C, Trolox and some amino acid derivatives were compared, the values for k_{Δ}^Q were in agreement with the values of their respective E_{ox} .⁹⁰ Bisby and coworkers⁹⁰ also demonstrated a pH-dependent effect, observing that the basic form of the above compounds exhibited values for the k_{Δ}^Q of 2–3 order of magnitude higher. In the above studies physical quenching prevailed over chemical quenching, but the ratio between both rate constants $k_{\Delta,ph}^Q$ and $k_{\Delta,r}^Q$ is known to depend significantly on the polarity of the solvent and the pH of the medium.^{96–98}

The third deactivation process corresponds with the electronic-to-vibrational energy transfer from $^1O_2$ to the solvent molecules. The water molecules of the aqueous medium and the buried methylene groups of lipids in membranes are the cellular components that mainly contribute to the deactivation of $^1O_2$ by electronic-to-vibrational energy transfer in biological systems. The k_{Δ}^Q depends notably on the energy of the highest stretching mode of the O–H or C–H bonds and range between 10^2 – 10^4 $M^{-1} s^{-1}$. Although they are rather small, the concentration of water in the aqueous medium and densely packed methylene groups in membranes is in a molar range. This results in a lifetime for $^1O_2$ in the absence of Qs (τ_{Δ}^0) of approximately 4 μs in neat water⁹⁹ and between approximately 7–30 μs in lipid membranes or vesicles.^{100,101} The mechanism can be understood as an exchange energy-transfer (or Dexter) mechanism, where the $^1O_2$ deactivation occurs after the formation of an encounter complex between $^1O_2$ and the solvent molecule. The E_{Δ} is converted into vibration of O_2 and the solvent molecule. Hurst and Schuster¹⁰² summarized in short that the greater the energy of the highest-frequency vibrational mode (*i.e.* the stretching vibration of the terminal bond) of the solvent, the nearer to resonance is a $^1O_2$ vibrational transition with a larger Franck–Condon factor. Although Hurst and Schuster¹⁰³ only included the fundamental vibrational transition for the solvent to explain the correlation between the k_{Δ}^Q and the energy of the highest-frequency vibrational mode of the solvent, the participation of overtone excitations of the terminal bond was included later to fully describe $^1O_2$ deactivation by solvent molecules.¹⁰⁴

12.5.2. Chemical Quenching: β -Carotene Products as Signaling Molecules

Car molecules are by far the most efficient physical Qs of $^1\text{O}_2$. However, chemical quenching of $^1\text{O}_2$ by Car molecules can be observed in solution, although it was first considered a very minor side effect in organic solvents ($k_{\Delta,ph}^Q \gg k_{\Delta,r}^Q$).⁸⁵ The oxidation of Car molecules such as β -Car by $^1\text{O}_2$ can be relatively significant in some mixtures of organic solvents and model membranes,^{105,106} where it has been estimated that one molecule of β -Car can deactivate about 1000–10 000 molecules of $^1\text{O}_2$ before it irreversibly becomes oxidized. This result might be of little relevance in *in vitro* studies, but when a Car molecule is part of a photosynthetic complex its chemical oxidation might limit its photoprotection role and consequently it can enhance the photodamage to the photosynthetic complex. The photoinduced oxidation of β -Car by $^1\text{O}_2$ in organic solutions mainly yields a mixture of β -apo-carotenal products and β -ionone together with the 1,4-cycloaddition product β -carotene-5,8-endoperoxide, which fades out if the photo-oxidation treatment persists.^{107–110} On the contrary, β -carotene-5,8-endoperoxide is the main photo-oxidation product in model membranes and methyl linolate solutions.^{106,111} Similar oxidation products were found when Zea and Lut were photo-oxidized in the presence of $^1\text{O}_2$, but the ratio between them and the time needed for their accumulation varied among Xan molecules.¹⁰⁹ In the above studies, there was no evidence for the formation (except possibly in trace amounts) of epoxide products, suggesting that β -Car and Xan molecules mainly followed a direct photo-oxidation by $^1\text{O}_2$ and that the free-radical oxidation of β -Car and Xan had little contribution.

The PSII RC is a special case where different oxidation products of β -Car can be observed depending on whether the electron chain reaction is inhibited on the donor side or acceptor side of PSII. After charge separation, P_{680}^{+} is reduced by Tyr_Z, which subsequently oxidizes the Mn cluster of PSII, the site of the catalytic water oxidation. In the event that the electron transport from the donor side of PSII is impaired and P_{680}^{+} is not reduced by Tyr_Z, a secondary electron-donation pathway takes over to protect the PSII from oxidative damage induced by P_{680}^{+} ($E_{\text{red}} \sim 1.1$ V). In this case, the cytochrome b_{559} , Chl_{zD2} and β -Car_{D2} act as secondary electron donors;¹¹² Chl_{zD1} and β -Car_{D1} can also act as secondary electron donors in the event that the accessory pigments of the D2 protein are removed.³⁰ As a result of the donor side photoinhibition, β -Car_{D2} is oxidized by P_{680}^{+} and the radical cation of β -Car is formed. If the illumination of the PSII RC persists in the presence of electron acceptors, the two β -Car molecules undergo an irreversible photobleaching.¹¹³ The final products were not β -Car epoxides and $^1\text{O}_2$ was not responsible for the β -Car oxidation products. If, instead, the acceptor side of PSII is inhibited, $^1\text{O}_2$ is formed. The two β -Car molecules, though unable (on account of lacking van der Waals contact with Chl_{D1} and P_{680}) to prevent the sensitization of $^1\text{O}_2$ by quenching the sensitizer, can still quench $^1\text{O}_2$ as it diffuses out of the protein matrix of the PSII RC. Such a role is played by both β -Car molecules, as was

demonstrated in preparations of PSII RC complexes containing one or two molecules of β -Car.³⁴ However, random diffusion of $^1\text{O}_2$ inevitably damages the complex.

Although the physical quenching of $^1\text{O}_2$ by the two β -Car molecules in the PSII RC is not questioned, high-light treatments in leaves of *Arabidopsis* have shown that oxidation products of β -Car molecules can accumulate over time.¹⁰⁹ An analysis of such oxidation products revealed that β -carotene-5,8-endoperoxide levels increased rapidly. Ramel and coworkers¹⁰⁹ established that the formation of β -carotene-5,8-endoperoxide was associated with $^1\text{O}_2$ photosensitization in the PSII, implying that the chemical quenching of $^1\text{O}_2$ by β -Car, not some enzymatic oxidation or a reaction with another type of ROS, was responsible for the formation of this β -Car oxidation product. Together with β -carotene-5,8-endoperoxide, a variety of volatile short-chain oxidation products of β -Car such as the ketone β -ionone and the aldehyde β -cyclocitral were also formed after high-light treatments *in vivo*.^{109,114} Interestingly, β -cyclocitral, but not β -ionone, induced the expression of specific markers for $^1\text{O}_2$ and also activated defence responses, while at the same time it repressed the expression of transcripts related to plant development and biogenesis. In this respect, volatile short-chain oxidation products of β -Car, particularly β -cyclocitral, were proposed to play a role similar to that carried out by other reactive electrophilic species such as secondary end products of lipid peroxidation, acting as sensing and signaling molecules that can reprogram the gene expression of plants.^{110,114}

12.5.3. Lipophilic Quenchers: Can α -Tocopherol Outperform β -Carotene in Photoprotecting PSII RC?

Tocopherols are lipophilic antioxidants that are restricted to the lipid matrix of biological membranes, where they usually exhibit very little mobility and form aggregates at high concentrations.¹¹⁵ It is well established that tocopherols, by quenching lipid peroxyl radicals, prevent the propagation of lipid peroxidation in membranes. They act by donating the hydrogen atom of its hydroxyl group to the lipid peroxyl radical. The tocopheroxyl radical is then recycled in the presence of antioxidants such as ascorbic acid and GSH, being again ready to donate the hydrogen atom.¹¹⁶ Additionally, tocopherols can quench $^1\text{O}_2$, although the fate of the tocopherols molecules and the ability for physical or chemical quenching depend on the type of tocopherol (*i.e.* α , β , γ or δ) and the chosen solvent.^{96,117} The role of tocopherols, particularly α -tocopherol, as a Q of $^1\text{O}_2$ in thylakoid membranes has been extensively studied over the last decade. This antioxidant was proposed to play the photoprotection role that the β -Car molecules cannot play efficiently in the PSII RC.^{118,119} Using the herbicide pyrazolynate, an inhibitor of the 4-hydroxyphenylpyruvate dioxygenase belonging to the biosynthetic pathway of tocopherol and plastoquinone, Trebst and coworkers¹²⁰ observed a decrease in the tocopherol content when the alga *Chlamydomonas reinhardtii* was subjected to high light stress, together with a concomitant loss of the D1 protein of PSII.

This was supported by other experiments involving the double mutant *vte1 npq1* of *Arabidopsis*, which is deficient in the biosynthesis of both tocopherol and Zea.¹²¹ Nearly half of the D1 protein was lost when the plants were exposed to high light levels at low temperatures. In both experiments, it was proposed that $^1\text{O}_2$ produced by the PSII RC was responsible for the photodamage to the D1 protein. In further studies, it was demonstrated that α -tocopherol deficiency in the mutants *vte1* of *Arabidopsis* and *slr0090* of *Synechocystis* sp. PCC6803 enhanced the susceptibility of PSII to photoinhibition; however, it was proposed that the repair cycle of the photodamaged PSII was inhibited, while the rate of photoinactivation of PSII was not affected.^{122,123} The inhibition of *de novo* synthesis of the D1 protein was investigated in detail by Nishiyama and coworkers^{124,125} and they suggested that the action of ROS such H_2O_2 or $^1\text{O}_2$ was associated with the specific inactivation of an elongation factor, impairing protein biosynthesis. In order to clarify whether the D1 protein bound to the PSII RC could be photoprotected by α -tocopherol from $^1\text{O}_2$, preparations of PSII RC were subjected to high light levels in the presence of Trolox.³¹ When Trolox was solubilized in the detergent micelles containing the PSII RC, it was observed that Trolox could deactivate (mainly by chemical quenching) $^1\text{O}_2$ diffusing out of the protein matrix. However, Trolox was unable to photoprotect both the pigments of the PSII RC and the membrane regions of the D1 and D2 proteins, although a partial photoprotection was apparent for the surface-exposed regions of the D1 and D2 proteins.³¹ All this suggests that the protein matrix of the RC is itself a barrier that does not allow Qs of $^1\text{O}_2$ to cross it. By extending this conclusion to other studies carried out with α -tocopherol, it was proposed that α -tocopherol cannot outperform the photoprotection role of β -Car in the PSII RC and that α -tocopherol can only quench $^1\text{O}_2$ that escapes from the RC and diffuses within the thylakoid membranes. Although it is generally accepted that the chemical quenching of $^1\text{O}_2$ by α -tocopherol leads to the irreversible formation of α -tocopherolquinone,⁹⁷ it has been recently proposed that α -tocopherol can be recycled from the primary α -tocopherol oxidation product (*i.e.* 8a-hydroperoxy- α -tocopherone) in thylakoid membranes of *Chlamydomonas reinhardtii* at high-light conditions while low pH is kept in the lumen of thylakoids.¹²⁶

In addition to α -tocopherol, new lines of evidence have shown that plastoquinol molecules can quench $^1\text{O}_2$ produced by the PSII RC.¹²⁷ Interestingly, plastoquinol has been demonstrated to be more active than α -tocopherol in the quenching of $^1\text{O}_2$ produced by *Chlamydomonas reinhardtii* at high light conditions.¹²⁶ Photo-oxidation of plastoquinol newly produces plastoquinone that can be recycled and can be newly active in the electron-transport chain of PSII. The simultaneous accumulation of plastoquinone C—a hydroxyl derivative of plastoquinone with the hydroxyl group in the side chain—was regarded as an indicator of the oxidation level by $^1\text{O}_2$.^{126,128} The reaction of plastoquinol and $^1\text{O}_2$ can thus be understood as a photoprotection mechanism of PSII during high light stress to avoid the accumulation of the plastoquinone pool in an over-reduction state that might enhance $^1\text{O}_2$ production and so induce a more severe photodamage.¹²⁹ Other prenyllipids

such as plastochromanol found mainly in seeds, but also at low levels in leaves of plants, have also been shown to play a role in the quenching of $^1\text{O}_2$.¹³⁰ The reaction between plastochromanol and $^1\text{O}_2$ yields hydroxyl-plastochromanol, a product that is observed even at very low light conditions.¹²⁸ In conjunction with the quenching family of prenyllipid compounds in thylakoids, polyunsaturated fatty acids have also been suggested to play a role in the quenching of $^1\text{O}_2$ in membranes. However, if the k_{Δ}^Q for polyunsaturated fatty acids is compared with that of other Qs it becomes clear that their photoprotection role is of little physiological relevance.¹³¹

12.5.4. Hydrophilic Quenchers

Vitamin C is the major antioxidant in plants and can reach concentrations between 20–300 mM depending on the plant species and light conditions.⁹¹ The photoprotection roles of ascorbate in photosynthesis as enzymatic antioxidant, direct radical Q of α -tocopheryl radicals or cofactor of the Vio de-epoxidase require it as an electron donor, resulting in the production of the monodehydroascorbate (MDHA) radical. MDHA is a radical of relatively high stability and is directly recycled to form ascorbate by NADPH- or ferredoxin-dependent enzymatic reduction or can disproportionate to ascorbate and dehydroascorbate (DHA), the latter being transformed into ascorbate by GSH-dependent enzymatic reduction.^{91,132} Apart from this enzymatic antioxidant role, vitamin C can directly react with $^1\text{O}_2$ producing DHA and H_2O_2 .¹³³ The reaction transforms $^1\text{O}_2$ with high reactivity and very short τ_{Δ} in another type of ROS characterized by having a longer τ and greater diffusion mobility. The $^1\text{O}_2$ -dependent consumption of reducing equivalents of NADPH, ferredoxin or GSH to detoxify H_2O_2 and to regenerate ascorbate can thus contribute indirectly to the regulation of the redox state balance of chloroplasts. The application of PSs of $^1\text{O}_2$ to discs of plant leaves has been demonstrated to alter the concentration of ascorbic acid and GSH in the light.¹³⁴ The concentration of ascorbate markedly decreased, but in contrast the concentration of GSH increased for most of the PSs, suggesting there was a compensating effect for the low content of ascorbate. A similar compensation was described for *vtc2*, an ascorbates-deficient mutant of *Arabidopsis*.^{135,136} When the cellular distribution of ascorbate and GSH was investigated in the *vtc2* mutant, a six-fold increase in the concentration of GSH was observed in chloroplasts, together with an accumulation of ascorbic acid in the thylakoid lumen.¹³⁷ In spite of the increase in GSH concentration in *vtc2*, this mutant exhibits high levels of lipid peroxidation,¹³⁸ suggesting that GSH cannot fully counterbalance the loss of ascorbate. GSH is known to be an efficient Q of $^1\text{O}_2$ like other sulfur-containing compounds.^{139,140} The quenching of $^1\text{O}_2$ by GSH is mainly chemical and pH dependent¹⁴⁰ and its k_{Δ}^Q is similar to that of ascorbate.⁹⁰ Ascorbate is much more abundant than GSH in chloroplasts⁹¹ and the increase in GSH concentration in *vtc2* might not thus be high enough to compensate the antioxidant effect of ascorbate.

Vitamin B₆ is a collective term that embraces six interconvertible pyridine derivatives (*i.e.* pyridoxine, pyridoxal, pyridoxamine and their phosphorylated forms) and plays a role as a cofactor of a large number of enzymes with remarkably different activities, although they conserve unique regions involved in the binding of the vitamers.¹⁴¹ Together with its role as an enzymatic cofactor, vitamin B₆ has the ability to quench ¹O₂ *in vitro*.¹⁴² The analysis of k_{Δ}^Q in organic solutions and pH-buffered water solutions indicates that vitamin B₆ must mainly deactivate ¹O₂ by chemical reaction in biological systems. The addition of vitamin B₆ to protoplasts of the conditional *flu* mutant of *Arabidopsis* having high concentrations of Pchl_{id} protects them from cell death during illumination.¹⁴³ The *in vivo* photoprotection role of vitamin B₆ was also investigated in the *pdx1* mutants of *Arabidopsis* characterized by having defects in the vitamin B₆ biosynthesis.¹⁴⁴ Together with a low accumulation of vitamin B₆, the *pdx1.3* mutant also contains a lower content of Chl and smaller antenna complexes, but the photosynthetic activity was not significantly different from that in wild-type plants. When the mutant was exposed to high light levels, it showed a marked decrease in the tolerance to photo-oxidative stress, which was accompanied by an increase in ¹O₂ production and lipid peroxidation and a decrease in tocopherol. Lipid peroxidation was more severe when the *pdx1* mutant was crossed with the double mutant *vte1 npq1* deficient in vitamin E and Zea. The leaves of the triplet mutant bleached at high light, suggesting an interplaying function between vitamin B₆, vitamin E and Zea in photoprotection. Titiz and coworkers¹⁴⁵ analyzed the amount of the D1 protein in the *pdx1* mutants at low and moderate light conditions and they established that the D1 protein was photodamaged at moderate light conditions, whereas the PSII of wild-type plants did not exhibit any apparent photoinhibition. Titiz and coworkers¹⁴⁵ concluded that there might be a pool of vitamin B₆ close to PSII for an efficient quenching of ¹O₂, although they did not rule out that the photosensitivity of the *pdx1* could also be due to a lower turnover of Chl because of the requirement of vitamin B₆ as a cofactor of some of the enzymes involved in Chl biosynthesis. The analysis of the cellular content of vitamin B₆ led Havaux and coworkers¹⁴⁴ to suggest that vitamin B₆ is uniformly distributed between chloroplasts and the rest of the cell, and that the pool of vitamin B₆ located in the cytosol could quench ¹O₂ that “escapes” from chloroplasts.

12.6. Singlet Oxygen Diffusion in Plants

12.6.1. Can Singlet Oxygen Be a Signaling Molecule Itself Based on Its Diffusion Distance?

The intracellular diffusion distance by ¹O₂ is a matter of scientific interest. It is not only a question about how far it can go, but also if ¹O₂ itself can be responsible for the activation of defence responses in plants. An estimate for the intracellular diffusion coefficient of ¹O₂ was given for HeLa cells.¹⁴⁶

In these cells, the diffusion coefficient of O_2 was proposed to be ~ 5 – 10 times lower than that in neat water based on a comparative analysis with sucrose solutions, where the viscosity of the solution changed with the sucrose concentration. The absolute value of the apparent intracellular diffusion coefficient (D) was thus estimated to be ~ 2 – $4 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. Similarly, the diffusion coefficient for O_2 in lipid membranes was reported to range between 1.2 – $1.8 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$.^{100,147} Even lower values were reported for the diffusion coefficient of O_2 in several biological systems.¹⁰⁰ In particular, a value of $\sim 7 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ was given for the diffusion coefficient of O_2 in chloroplast, where in addition τ_Δ was estimated to be as short as $0.07 \mu\text{s}$.^{6,100}

The extremely short τ_Δ in chloroplasts is due to the presence of lipophilic Qs such as Car, Chl, vitamin E molecules in thylakoid membranes and hydrophilic Qs such as vitamin C, B_6 and GSH molecules in the stroma (Table 12.1). Likewise, other electron-enriched compounds such as polyunsaturated acids and amino acid residues of the protein matrix of membrane-embedded photosynthetic complexes and water-soluble proteins can react with $^1O_2$ and so contribute to the shortening of τ_Δ in chloroplasts. Accepting the small diffusion coefficient of O_2 in chloroplast thylakoids determined by Krasnovsky,^{6,100} the molecules of $^1O_2$ could simply diffuse a distance, $d = (6Dt)^{1/2}$, of $\sim 60 \text{ \AA}$ during a time span equivalent to τ_Δ , which corresponds with the width of the thylakoid membranes. In this case, $^1O_2$ would be deactivated mostly in the place of generation. This result is in agreement with the proposed geminate quenching of $^1O_2$ by RC type II.^{35,148} If instead we make use of the diffusion coefficient for O_2 in lipid membranes,^{100,147} the distance that $^1O_2$ could diffuse in thylakoid membranes is $\sim 30 \text{ nm}$ and then it could certainly leave the thylakoid membranes.

The k_Δ of $^1O_2$ produced by the PSII RC in neat water was about $3 \mu\text{s}$,³⁵ a value similar to that of $^1O_2$ in the nucleus of HeLa cells, where it was proposed that DNA is not a particularly good Q of $^1O_2$.¹⁴⁹ However, as said above, this cannot be the case for chloroplasts where high levels of efficient hydrophilic Qs of $^1O_2$ are present in the chloroplast stroma. In the event that $^1O_2$ escapes from PSII and it diffuses out of the thylakoid membrane, it will encounter a very viscous medium, where the protein concentration of the chloroplast stroma can reach values close to 300 mg ml^{-1} . At the same time, $^1O_2$ will encounter

Table 12.1. Some physical and chemical quenchers of $^1O_2$ in chloroplasts.

Q	$k_\Delta^Q, \text{M}^{-1} \text{s}^{-1}$	[Q]
Car	$\sim 10^8$ – 10^{10}	~ 20 (T) ^a , ~ 6 – 12 (E) ^b $\mu\text{g mg}^{-1}$ prot.
Chl	$\sim 10^7$ – 10^8	~ 160 (T) $\mu\text{g mg}^{-1}$ prot.
α -Toc	$\sim 10^8$	~ 1 – 2 (T), ~ 3 – 8 (E), $\mu\text{g mg}^{-1}$ prot.
Asc	$\sim 10^8$	$\sim 10^{-2}$ – 10^{-1} M
GSH	$\sim 10^8$ – 10^9	$\sim 10^{-3} \text{ M}$
Vit. B_6	$\sim 10^8$	$\sim 10^{-3} \text{ M}$
C-H, O-H	$\sim 10^2$ – 10^4	$\sim 10^0$ – 10^1 M

^aT stand for thylakoid (see ref. 151 for further details).

^bE stand for envelope (see ref. 151 for further details).

hydrophilic Qs such as vitamin C, vitamin B₆ and GSH with k_{Δ}^Q of the order of $10^8 \text{ M}^{-1} \text{ s}^{-1}$ and in concentrations that go from $\sim 10^{-3} \text{ M}$ for nonphosphorylated vitamin B₆ and GSH to $\sim 10^{-2}$ – 10^{-1} M for vitamin C.^{90,91,139,140,142,144} To put it simply, if one only takes into account, first, the lowest concentration of the most quoted concentration range of ascorbate in chloroplasts (20–50 mM), secondly, the value of $3.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for the k_{Δ}^Q of the quenching of $^1\text{O}_2$ by ascorbate and, thirdly, the upper estimated value of the apparent diffusion coefficient for O_2 in viscous intracellular medium ($\sim 2\text{--}4 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$), one can estimate that τ_{Δ} is $\sim 200 \text{ ns}$ in the chloroplast stroma and the distance that $^1\text{O}_2$ would diffuse out in this medium is only about 20 nm during a time span of $\sim 200 \text{ ns}$ or about 30 nm during two times τ_{Δ} (Figure 12.4). Again, the more favorable estimate for a larger diffusion distance puts $^1\text{O}_2$ deactivation in a radius similar to the longest dimension of the supramolecular complex of PSII.¹⁵⁰ If one assumes that $^1\text{O}_2$ is produced close to the chloroplast envelope, this also represents another barrier for $^1\text{O}_2$. The chloroplast envelope, which contains both Car and tocopherol molecules, has a concentration in Car and tocopherol that does not differ much from that in thylakoid membranes.¹⁵¹ Double sensor molecules containing 4-amino substituted 1,8-naphthalimide as a fluorophore and a sterically hindered amine (pre-nitroxide) or pyrroline nitroxide as a Q and radical capturing moiety have been synthesized and used to determine whether $^1\text{O}_2$ produced in chloroplasts can leave from them.¹⁵² However, the results did not show evidence for the detection of $^1\text{O}_2$ outside this organelle. All this suggests that $^1\text{O}_2$ photosensitized in chloroplasts is quenched in the interior of chloroplasts and

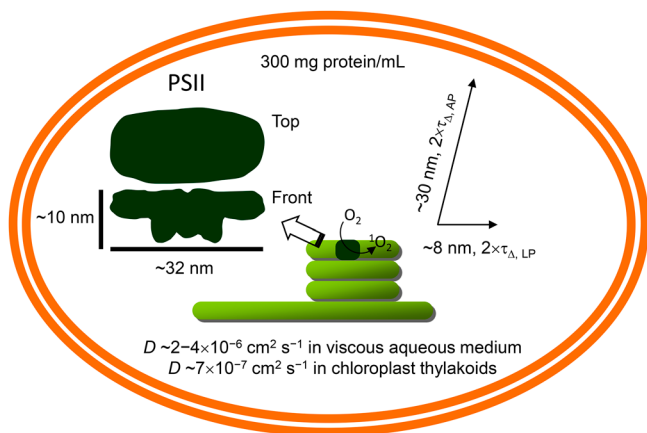


Figure 12.4. Schematic representation of the distance that $^1\text{O}_2$ photosensitized by PSII can diffuse out in the thylakoids (lipid phase, LP) and stroma (aqueous phase, AP) based on the diffusion coefficient of O_2 estimated in chloroplast thylakoids or viscous intracellular medium, the k_{Δ}^Q and the approximate concentration of Qs is given in Table 12.1. The distance is calculated for $2 \times \tau$ in order to compare it with the dimensions of the supercomplex PSII. See Section 12.6 and references therein for further details.

that the amount (if any) of $^1\text{O}_2$ that leaves chloroplasts must be insignificant. Consequently, it is hard to reconcile the role of $^1\text{O}_2$ itself as a signaling molecule with the facts that its diffusion is severely hampered inside chloroplasts by the viscous stroma and the high concentration of efficient lipophilic and hydrophilic Qs.

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Chapter 13

Genetically Encoded Singlet Oxygen Photosensitizers

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13.1. Introduction

The ability to selectively control the site of singlet oxygen ($^1\text{O}_2$) production in a cell is crucial for inflicting photodynamic damage in a desired location. While chemical functionalization of a photosensitizer (PS) can somewhat tune its intracellular location as a consequence of solubility and/or complex formation with a biomolecule,¹ nonspecific binding is difficult to avoid and can lead to uncontrolled photodamage. The use of PS–antibody conjugates does confer improved specificity;^{2–4} however, this approach needs the generation of the target antibody and its conjugation with the PS, and still suffers from nonspecific antibody binding. In another approach, genetic tags with tetracysteine motifs that bind biarsenical PSs,⁵ as well as other chemical tags^{6,7} have also been used, but these methods still need the exogenous addition of the PS, which does not completely solve the issue of nonspecific binding. Therefore, fully genetically encoded PSs, which can be fused to virtually any protein and are expressed in a cell without the need to add any external cofactors, are the way forward to achieve the best target specificity and thus provide absolute control of the $^1\text{O}_2$ production site.

Fluorescent proteins derived from *Aequorea* jellyfish and other marine organisms have been used since the 1990s as fully genetically encoded labels and probes. Some early work was carried out to investigate the photosensitization of reactive oxygen species (ROS) by the green fluorescent protein (GFP) in the context of photobleaching in fluorescence microscopy, and small amounts of $^1\text{O}_2$ were detected by means of electronic spin resonance⁸ and by indirect methods.⁹ A few years later, the fluorescent protein KillerRed was specifically evolved to efficiently generate ROS for its use in chromophore-assisted light inactivation (CALI) and other applications¹⁰ (see below and Chapter 35). Although it was later shown that KillerRed mainly produces other ROS (superoxide) and not $^1\text{O}_2$, it did bring the focus to the potential of fluorescent proteins as genetically encoded photosensitizers and has catalyzed the study of ROS photosensitization by fluorescent proteins at the molecular level. As we will discuss later, an increasing number of papers aim at providing a more detailed view of the mechanistic aspects of ROS photosensitization and the relation with the structure of these proteins.

More recently, miniSOG, another $^1\text{O}_2$ photosensitizing protein not structurally related to GFP, has been engineered from a phototropin photoreceptor.¹¹ MiniSOG and its variants have much higher $^1\text{O}_2$ photosensitization efficiency than GFP-like proteins, and thus promise to revolutionize all applications related to genetically encoded $^1\text{O}_2$ production.

Genetically encoded PSs have been used as tools to inflict damage at a specific cell location, in different variations that depend on the purpose of the damage: photodynamic therapy (PDT), photoablation, CALI and optogenetics. They have also been used as tools for different imaging modalities such as correlative light and electron microscopy (CLEM). In this chapter, we provide a molecular view of the photosensitization mechanisms and cover the main applications of the more significant genetically encoded PSs.

13.2. Photosensitization by GFP-Like Proteins

GFP is a fluorescent protein naturally present in *Aequorea victoria* jellyfish. It is formed by 238 amino acids arranged in an eleven-stranded β -barrel (one large β -sheet) and an α -helix penetrating through the cylinder that hosts a chromophore approximately in the middle of the cavity. The GFP chromophore, *p*-hydroxybenzylideneimidazolinone (HBDI), is autocatalytically formed from a tripeptide, typically Ser65–Tyr66–Gly67, in a multistage maturation process that requires the presence of molecular oxygen (Figure 13.1).

Early studies on $^1\text{O}_2$ formation by irradiated GFP appeared in the early 2000s. Electron spin resonance measurements and assays using sodium azide, a specific quencher of $^1\text{O}_2$, demonstrated that photobleaching of GFP and enhanced (E)GFP is in part a consequence of their oxidation by self-sensitized $^1\text{O}_2$.^{8,9} A few years later, $^1\text{O}_2$ photosensitization by EGFP was directly observed for the first time by time-resolved detection of NIR phosphorescence at 1270 nm.¹² This study revealed some interesting information about the effect of the protein environment of the chromophore on the kinetics of $^1\text{O}_2$

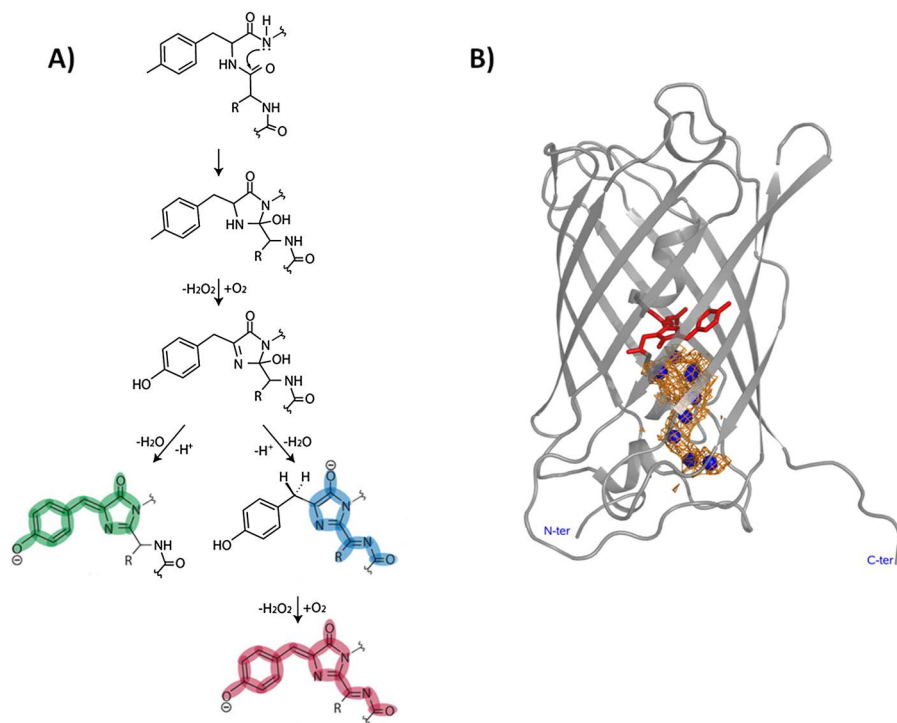


Figure 13.1. (A) Chromophore formation in GFP-like proteins (adapted from ref. 82). (B) X-ray crystallographic structure of a KillerRed monomer. The backbone is represented in gray, the chromophore in red, the cavity forming the channel is shown as orange and water molecules in the channel are depicted as blue spheres (adapted from ref. 19).

formation and deactivation. The β -barrel structure hinders diffusion of molecular oxygen and thus its interaction with the chromophore. One consequence of this is that the triplet-state lifetime of EGFP is increased to 25 μ s compared to 3 μ s for synthetic (free) HBDI. In addition, the $^1\text{O}_2$ lifetime also decreases to 4 μ s in EGFP (compared to 20 μ s for HBDI), suggesting quenching by certain amino acids. Due to the low efficiency of photosensitization, this study was unable to quantify the quantum yield for $^1\text{O}_2$ production (Φ_Δ) for EGFP, but it was determined for free HBDI as 0.004.¹² While this value could be regarded as an upper limit or a reference for Φ_Δ in the context of GFPs, it is not directly comparable with that of the protein as there are important structural and photophysical differences between the free and the protein-embedded chromophore. While HBDI in solution shows efficient excited-state deactivation due to photoisomerization, torsional motion is highly restricted in the protein, leading to a very significant enhancement of its fluorescence quantum yield (Φ_F). It is worth noting that this situation differs from that of the flavoproteins (see below).

Photosensitized $^1\text{O}_2$ has also been detected from a red fluorescent variant of GFP, namely TagRFP.¹³ In red fluorescent proteins, the chromophore maturation entails the formation of an *N*-acylimine double bond $\text{N}=\text{C}$ and an extended π -conjugation system (Figure 13.1).¹⁴ TagRFP had shown a clear oxygen dependence on its photobleaching,¹⁵ which is unusual in fluorescent proteins and suggested the participation of self-sensitized ROS. Indeed, $^1\text{O}_2$ phosphorescence could be detected and it was possible to estimate a Φ_Δ value of 0.004. This value was measured by using the specific fluorescent probe singlet oxygen sensor green (SOSG) and represents a lower limit for Φ_Δ , as only those molecules that are able to escape the β -barrel can be detected.¹³ A short triplet state lifetime of 3 μ s was found for TagRFP, suggesting a higher oxygen diffusion across the β -barrel compared to EGFP. Interestingly, TagRFP lacks the water channel connecting the chromophore with the bulk (different to KillerRed, see below). It was therefore proposed that diffusion of $^1\text{O}_2$ could be facilitated by the presence of temporal permeable gates in the protein due to dynamical breathing,¹³ as reported for other GFP-like proteins.¹⁶

A prominent example of a GFP-like PS is KillerRed, which was evolved from the *Hydrozoan* chromoprotein anm2CP in 2006. KillerRed shows a 1000-fold stronger phototoxic effect upon green-light irradiation than homologous GFP-like proteins.¹⁰ At physiological conditions KillerRed is formed by two 27 kDa monomers showing fluorescence excitation/emission maxima at 585/610 nm. Oxygen radicals (principally superoxide) and hydrogen peroxide have been detected as the main ROS products upon KillerRed irradiation^{17,18} by using a free-radical fluorescent probe and electronic paramagnetic resonance.¹⁸ The mechanism for superoxide radical formation would consist in direct electron transfer from the excited-state chromophore to molecular oxygen,¹⁹ which would in turn be (at least partially) dismutated into molecular oxygen and hydrogen peroxide.¹⁸ While this protein produces mainly other ROS instead of singlet $^1\text{O}_2$,^{17,18} it is pertinent to discuss its structural features and how they may relate to its ROS production in terms of access of molecular

oxygen and escape paths for ROS. The structural reasons for the mechanism of KillerRed phototoxicity are still unclear, but crystallographic data have provided some interesting insight. KillerRed monomers keep structural similarities with the rest of GFP-like proteins, folding in the typical β -barrel structure. The chromophore results from an autocatalytic maturation of residues Gln65–Tyr66–Gly67, and some surrounding amino acids play a potentially important role. For example, residues Glu68, Asn145, Thr201 and Glu218 have been suggested to stabilize the chromophore excited state and/or participate in the photoinduced electron-transfer reaction.^{19,20} The most exceptional feature of KillerRed is a long water-filled channel that allows the chromophore to be exposed to solvent molecules (Figure 13.1(B)).^{19,20} Although this channel has been observed in other nonphototoxic FPs,^{21–23} it has been noted that in KillerRed the channel has access to the most reactive part of the excited chromophore (*i.e.* the exocyclic double bond and imidazolinone moiety).²⁴ The ordered water channel in KillerRed therefore seems to be greatly responsible for its phototoxicity by connecting the external solvent with the protected chromophore and facilitating the diffusion of both molecular oxygen and photoinduced ROS in and out of the β -barrel, respectively.^{16,19,20} Residue Pro192 in the channel may gate the solvent flow to/from the channel.^{10,19} Additionally, KillerRed shows a smaller water pore at the β -barrel surface, which could also facilitate the entrance of molecular oxygen,^{21,25,26} but the contribution of this feature to the overall phototoxicity seems to be small.¹⁶ Therefore, the strong phototoxicity of KillerRed seems to be due to a combination of its exceptional structural properties: (i) a long water-filled ordered channel, which facilitates the access of solvent to the most reactive groups of the chromophore; and (ii) a precise amino acid configuration able to stabilize the chromophore in its excited state.

Another photosensitizing protein, SuperNova, has been evolved recently from KillerRed.²⁷ SuperNova retains KillerRed's ability to generate ROS but mutagenesis of six residues has rendered the new protein monomeric. This is important for proper fusion protein localization in cells, and it overcomes a drawback of KillerRed for its application in CALI. As mentioned above, the Asn145 residue in KillerRed was previously considered to be indispensable for the phototoxicity.¹⁰ However, the Asn145Ser mutation does not affect the phototoxicity of SuperNova, which shows equivalent photosensitizing activity in eukaryotic and prokaryotic systems to KillerRed.

13.3. Photosensitization by Flavoproteins

Flavoprotein is the term referring to those proteins harboring riboflavin derivatives as a prosthetic group (Figure 13.2). This family of compounds is involved in a wide array of biological processes, including photosynthesis, DNA repair and bioluminescence among others.^{28–30} The photophysical behavior of flavoproteins varies widely depending on the specific flavin cofactor (Table 13.1) and the amino acid residues that surround it.

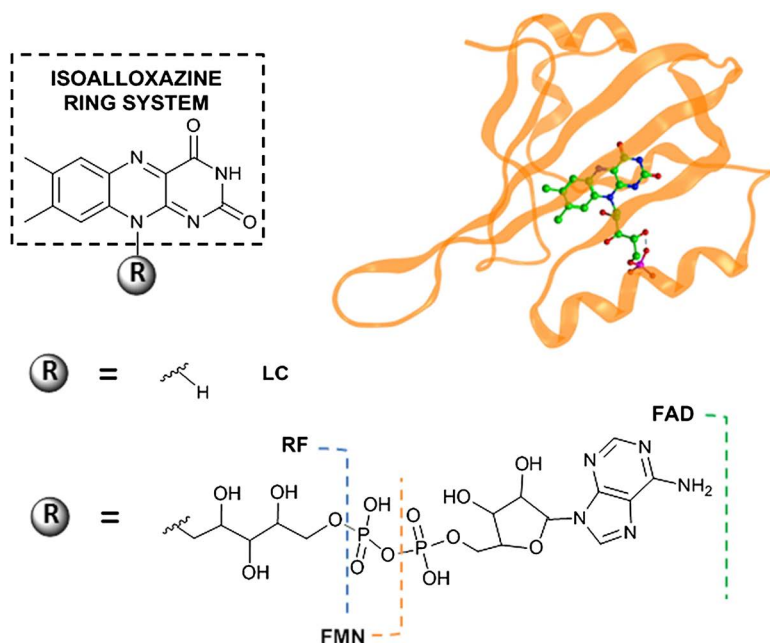


Figure 13.2. Flavin 7,8-dimethyl-10-alkylisoalloxazine ring core, main flavin derivatives and flavoprotein structure. Riboflavin (RF), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and lumichrome (LC).

Table 13.1. Photophysical properties of flavin derivatives and LOV-based FbFPs.

	Φ_F	Φ_T	Φ_A	Ref.
RF	0.26	0.61	0.51	42,45–47
FAD	0.04	0.15	0.07	42,48,49
FMN	0.25	0.60	0.51	42,48,50
LC	<0.10	0.71	0.36	46 and 51
MiniSOG	0.41	0.30	0.03	11,43,44
SOPP	0.45	0.30	0.19	52
Pp2 L30M	0.25	0.30	0.09	53 and 54

In solution, most flavins are endowed with mild blue fluorescence ($\Phi_F = 0.2 - 0.3$) and undergo intersystem crossing (ISC) quite efficiently ($\Phi_{ISC} = 0.3 - 0.7$), which in turn is reflected in relatively high yields of $^1O_2$ production.³¹

These properties may dramatically change when the flavin is bound to the protein. A good example of this can be found in phototropins, which are flavoprotein photoreceptors that mediate phototropism responses in plants and bacteria.^{31–33} Phototropins contain the so-called LOV (light, oxygen, voltage) domains in which the excited state of FMN rapidly forms a reversibly covalent bond with an adjacent Cys residue, which subsequently mediates further signaling.³⁴

Flavoproteins have been exploited in biotechnology due to the tunability of their optical properties when replacing specific amino acids. Mutagenesis of the reactive cysteine has been performed to engineer flavin-binding fluorescent proteins (hereafter FbFPs),^{34,35} leading to families such as EcFbFp, iLOV or PpFbFP.^{36,37,38} FbFPs are useful tags for fluorescence imaging that are able to outperform their GFP counterparts in some applications.^{38–40} The main advantages of FbFPs over GFPs are their small size (approximately 15 kDa), insensitivity to pH and the fact that they do not need molecular oxygen to mature.³⁶ On the other hand, brightness and photostability are not as good as in GFPs.

Rescuing the inherent $^1\text{O}_2$ photosensitizing ability of the flavin chromophore in FbFPs has been sought as a goal for advanced imaging techniques and cell-ablation purposes. The first successful attempt was presented in 2011 and named miniSOG (for mini singlet oxygen generator).¹¹ MiniSOG is a LOV-based flavoprotein 5 mutations away from the LOV2 domain of *Arabidopsis thaliana* phototropin 2.¹¹ As with other FbFPs, directed mutagenesis eliminated the conserved cysteine residue and included the Asp to Ser mutation, which had proven significant to photostability.⁴¹ MiniSOG presents blue absorption (maximum at 448 nm with a shoulder at 473 nm) and green emission (maxima at 500 and 528 nm) with the FMN prosthetic group tightly – but not covalently – bound to its pocket.¹¹ MiniSOG was originally reported to exhibit a Φ_{Δ} value of 0.47,¹¹ very close to that for free FMN in solution ($\Phi_{\Delta} = 0.51$).⁴² This value was later reassessed as $\Phi_{\Delta} = 0.03$ both by direct and indirect methods.^{43,44} Although relatively low compared to organic PSs, this value was at the time a record for fluorescent proteins.

Several hypotheses have been put forward to explain the revised Φ_{Δ} value, unexpectedly low compared to FMN. One possibility is that a substantial fraction of the nascent $^1\text{O}_2$ molecules is rapidly quenched as they diffuse through the protein. Specifically, typical $^1\text{O}_2$ quenching amino acids such as Tyr, Trp, His and Met can be found in miniSOG.⁴³ In this line, a recent molecular dynamics study suggests that Tyr30 and His85 may be mostly responsible for $^1\text{O}_2$ quenching.⁵⁵ It has also been proposed that electron transfer between the triplet excited state of FMN and vicinity amino acids may compete with the type-II $^1\text{O}_2$ production, leading to the formation of other ROS *via* type-I reaction.⁴⁴ Both mechanisms may potentially coexist given the rich photochemistry of FMN. Additionally, it was found that cumulative irradiation of miniSOG produced a 10-fold enhancement in its Φ_{Δ} value,⁴³ which could be due to the gradual photo-oxidation of residues involved either in quenching and/or electron-transfer processes. The Φ_{Δ} enhancement upon irradiation may explain the fact that miniSOG outperforms other tags for CLEM, in spite of having similar Φ_{Δ} value,⁵⁶ and may be used to optimize sample preparation protocols.

Most recently, rational mutation of miniSOG residues involved in hydrogen bonding with encased FMN has been explored. Out of the four studied positions, a single replacement of glutamine in position 102 (103 according to the original miniSOG numbering) for a leucine decreased the extent of

Table 13.2. Chromophore loading comparison between miniSOG and Pp2FbFP L30M.⁵⁵

	FMN	FAD	RF	LC
miniSOG	83%	2%	0%	15%
Pp2FbFP L30M	46%	41%	2%	11%

hydrogen bonding-mediated deactivation of the FMN excited state leading to a Φ_{Δ} value of 0.19 in aqueous solution and 0.25 in heavy water, six and eight times higher than the parent miniSOG, respectively.⁵² In addition to $^1\text{O}_2$, this mutant still produces superoxide, which highlights the challenge of specific ROS production. This work constitutes an important step towards understanding the factors affecting $^1\text{O}_2$ generation in flavoprotein photosensitizers.

Given the similarity of miniSOG to other FbFPs already reported,^{41,53} it was foreseeable that more members of the LOV-protein family would exhibit $^1\text{O}_2$ sensitizing properties. In a recent publication, the characterization of the $^1\text{O}_2$ photosensitization properties of the Leu30Met mutant of the Pp2FbFP from *Pseudomonas putida* has been provided.⁵⁴ This Met mutation exchange takes place in the highly conserved position 30, of great influence on the photochemical properties of LOV photoreceptors by affecting the stability of their photoadducts.^{57,58} The main result is that Pp2FbFP Leu30Met outperforms miniSOG by a three-fold factor, with a Φ_{Δ} value of 0.09 (Table 13.1). Besides being a more efficient $^1\text{O}_2$ generator, Pp2FbFP Leu30Met presents several particularities and differences compared to miniSOG. The most intriguing one is that, unlike miniSOG, transient absorption measurements revealed the existence of two independent triplet states, each one with distinct $^1\text{O}_2$ photosensitizing ability and oxygen accessibility.⁵⁴ These differences observed in the triplet-state behavior between the two flavoproteins have been ascribed to the presence of different flavin derivatives bound to Pp2FbFP Leu30Met (miniSOG almost exclusively binds FMN, see Table 13.2 and ref. 43 and 54). However, other possibilities such as dynamic protein conformation equilibrium or, less likely, the formation of asymmetric protein dimers, cannot be ruled out.

Since the protein structures of miniSOG and Pp2FbFP Leu30Met have not been solved so far and their sequence homology is low, it is difficult at this point to draw general conclusions about the relations between structure and photosensitizing properties in flavoproteins. However, they represent the most promising family to develop new and better genetically encoded $^1\text{O}_2$ PSs at the moment.

13.4. Applications of Genetically Encoded Photosensitizers

The applications that have derived from the availability of genetically encoded PSs take advantage of the full control on PS localization and can be broadly classified into two groups. The first group encompasses applications

in which the photosensitizing protein inflicts damage on its surrounding medium (typically another protein fused to it), for example in PDT,^{59–61} optogenetics and CALI (see Chapter 35). In addition, genetically encoded PSs are useful tools for advanced imaging technologies.

Early studies used $^1\text{O}_2$ generated by GFP in CALI experiments. This technique is employed to assess protein function in living cells by specifically inactivating the target protein through photochemical damage. It was possible to perform CALI using one-photon^{62,63} and two-photon⁶⁴ illumination of (E)GFP. However, these studies were limited by its poor efficiency of $^1\text{O}_2$ production. Development of improved sensitizing FPs such as KillerRed and miniSOG have enabled a wider range of CALI experiments and similar optogenetic applications, as discussed at length in Chapter 35.

The possibility to have a purely endogenous PS capable of sensitizing $^1\text{O}_2$ (and other ROS) at the desired localization has also led to interesting studies in PDT, providing novel mechanistic information of photoinduced cell death. On the one hand, the photosensitizing ability of KillerRed in eukaryotic cells has been studied on different cellular locations. High phototoxicity was observed when the protein was localized in mitochondria and plasma membrane; in contrast, a mild effect was effected when expressed in the cytosol.^{10,65} Further studies also demonstrated a very efficient photodamage when fused to histone H2A.⁶⁶ On the other hand, KillerRed is biodegradable, thus overcoming the long-lasting cutaneous photosensitivity and hyperpigmentation, which are the major side effects of externally administered PS.^{67–69} In another study, it was described that while miniSOG was more phototoxic than KillerRed *in vitro* it failed to kill cancer cells in tumor xenografts *in vivo*.^{68,70,71} The lack of FMN in such tissues as a consequence of poor vascularization has been suggested as the main explanation.⁷¹ Genetically encoded immunoconjugates have also been engineered including an antibody fragment fused to the protein sequence. *In vitro* results have successfully shown that the constructs preserve their functional properties leading to improved targeting and cell photokilling both with KillerRed^{17,72} and miniSOG.⁷⁰

Equivalent studies have also been performed in the context of antimicrobial PDT. The phototoxic properties of several monomeric and dimeric FPs expressed in the cytosol of *E. coli* have been tested, with KillerRed and miniSOG resulting, by far, the most efficient at inactivating bacteria.^{43,73,74} TagRFP also accomplished bacterial death by purely endogenously generated $^1\text{O}_2$, although high light doses were required due to its modest Φ_Δ value ($\Phi_\Delta = 0.004$).⁷⁵ This work also highlighted the power of genetically encoded PSs to unravel mechanistic aspects related to sensitized cell death. Damage extending from the inner to the outer membrane was evaluated upon irradiation of TagRFP or miniSOG expressed in the cytosol of *E. coli*, showing significant correlation between $^1\text{O}_2$ photoproduction and the extent of membrane damage.^{74,75} These experiments are harder to perform with standard organic PSs since it cannot be excluded that a fraction of sensitizing molecules will remain outside the bacterial cell wall.

As mentioned above, miniSOG was originally conceived as a tool for correlative light and electron microscopy (CLEM). CLEM combines the advantages of fluorescence microscopy (*e.g.* the ability to analyze processes in living cells, identify specific biomolecules, define areas of interest and give overviews of samples) with the high resolution obtained in the electron microscope, which is necessary for detailed localization and characterization of structures up to molecular dimensions. Combining these techniques, cells and tissues can be studied from the micrometer to nanometer scales.⁷⁶ The role of $^1\text{O}_2$ in CLEM is to locally polymerize 3,3'-diaminobenzidine (DAB) into an electron-dense precipitate visible by electron microscopy after staining with osmium^{77,78} (Figure 13.3). This reaction can also be initiated by a genetically encoded peroxidase and hydrogen peroxide, but the short lifetime and diffusion length of $^1\text{O}_2$ result in a much improved spatial resolution. In an advanced modality, electron tomography can be used in order to obtain 3D reconstruction of protein complexes.⁷⁷

Recent technological advances are leading to a revival of CLEM, including the availability of genetically encoded photosensitizer proteins that can be fused with a protein of interest. In spite of the low efficiency of ROS generation, GFP and yellow fluorescent protein (YFP), as well as enhanced variants (EGFP and ECFP), have been used for CLEM in early studies.^{77,79,80} More recently, engineered flavoproteins such as miniSOG have emerged as more promising alternatives due to their smaller size and higher $^1\text{O}_2$ quantum yield.¹¹ CLEM using miniSOG has made progress in understanding the role of α -synuclein accumulation in neurons affected by Parkinson's disease.⁵⁶

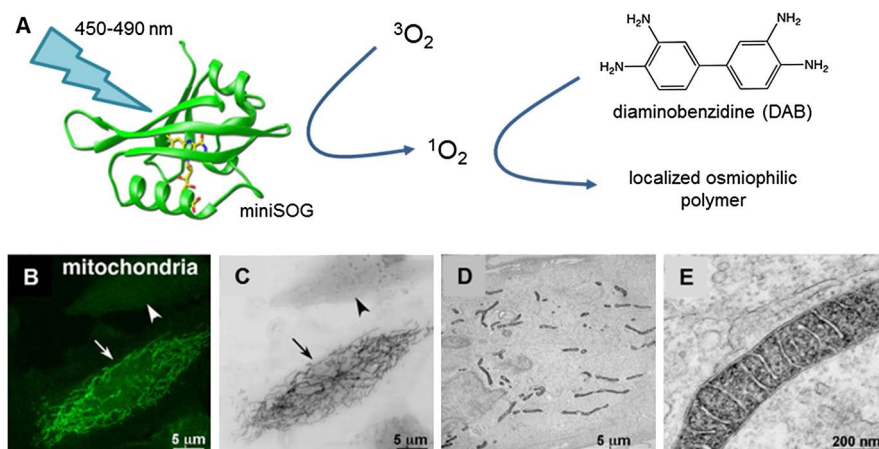


Figure 13.3. (A) Schematic diagram of how miniSOG produces EM contrast through $^1\text{O}_2$ generation upon blue-light illumination. Correlated confocal fluorescence (B), transmitted light after DAB photo-oxidation (C), and electron microscopic (D and E) imaging of mitochondrial targeted miniSOG. The differential contrast generated between a transfected (arrows) and nontransfected cell (arrowheads) is evident. Adapted from ref. 11.

MiniSOG has also been used in another imaging modality termed “singlet-oxygen triplet energy transfer-based” (STET) imaging.⁸¹ STET relies on the ability of $^1\text{O}_2$ to diffuse away from its site of generation and react at relatively remote sites, thereby extending the range of conventional “molecular rulers” such as FRET (Förster resonance energy transfer). In STET, two different proteins, which are expected to form a protein complex in a particular cellular process, are separately fused to a photosensitizer and a $^1\text{O}_2$ sensor, both genetically encoded. Irradiation of the photosensitizer generates $^1\text{O}_2$ that diffuses away until it encounters and reacts with the sensor. The enhancement of the sensor fluorescence directly depends on the distance at which $^1\text{O}_2$ is generated, such that the closer the two proteins are, the higher the amount of $^1\text{O}_2$ that reaches the sensor. In the core of the development of STET is the identification of the fluorescent protein IFP1.4 that seems to be suitable as a genetically encoded $^1\text{O}_2$ sensor.⁸¹

In contrast to FRET, STET can probe distances of up to tens of nanometers, which is particularly useful to study processes and structures of large protein complexes.

13.5. Conclusions and Outlook

Genetically encoded photosensitizers are the best possible tool for full control of the $^1\text{O}_2$ generation site. The complexity of proteins compared to organic molecules makes it challenging to rationalize the precise role of individual aminoacids and to draw a full picture of the relation between structure and ROS formation. However, progress has been made to understand some of the factors affecting photosensitization, in turn allowing to engineer mutants that photosensitize $^1\text{O}_2$ more efficiently. Another challenge that lies ahead is to better control the specificity in ROS generation, *i.e.* minimize superoxide formation. While this is not necessarily important to carry out certain biological experiments like cell ablation, it is crucial if genetically encoded photosensitizers are to be used in mechanistic studies to understand the role of singlet oxygen in cells.

Better genetically encoded photosensitizer proteins with higher efficiency and specificity of $^1\text{O}_2$ generation may improve techniques such as CALI, optogenetics and CLEM and allow new biological questions to be answered.

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Chapter 14

Singlet Oxygen Generation by Drugs and Their Metabolites

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14.1. Introduction

Photosafety is becoming an important issue to take into account in drug development. Indeed, modern lifestyle is associated with a higher exposure to sunlight and has been recognized as one of the factors responsible for increased skin photosensitization side effects, which result from interaction of UV-light with xenobiotics in the body. A deep knowledge of drug photo-reactivity is necessary to understand the photobiological properties and to anticipate the appearance of photosensitivity side effects.^{1,2} In this context, photochemists and photobiologists have been involved in a science-based approach for photosafety evaluation.

Photosensitization occurs through Type-I or Type-II processes. The former usually includes hydrogen-abstraction or electron-transfer processes and results in the production of radical species that can further lead to oxidation of biomolecules. The latter process is linked with singlet oxygen ($^1\text{O}_2$) generation and its attack to biological targets.

In the present chapter, the capability to photoinduce singlet oxygen is summarized for the most relevant families of drugs like nonsteroidal anti-inflammatory drugs (NSAIDs), antibacterial quinolones, statins and phenothiazines, among others. In some cases, the properties of drug photoproducts and metabolites are also given. However, the photosensitizers used for photodynamic therapy are not considered here, as their properties are sufficiently addressed in separate chapters.

Table 14.1 includes the quantum yields of singlet oxygen formation (Φ_Δ) as key data. Since the solvent is an important parameter, the Φ_Δ values are given both in non protic – generally acetonitrile – and protic – *i.e.* aqueous solution – media. In some cases, the photogenerated singlet oxygen is quenched (physical and/or chemical quenching) by the drug; the rate constant of this process is reported as k_q ($^1\text{O}_2$). Several methodologies have been employed in order to detect and quantify $^1\text{O}_2$ production. The most widespread and direct technique consists in the detection of characteristic emission at 1270 nm by means of time-resolved or steady-state near-infrared spectroscopy (Table 14.1, methodologies A and A', respectively).³ Formation of $^1\text{O}_2$ has also been evaluated by indirect measurements based on its chemical trapping. In this context, electron paramagnetic resonance (EPR) detection of the stable 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) radical, generated from 2,2,6,6-tetramethylpiperidine (TEMP) reaction with $^1\text{O}_2$, has been extensively used (methodology B).^{4,5} However, a limitation of this technique has been recently addressed by demonstrating that misleading formation of the TEMPO radical could be obtain from photoinduced electron transfer between the photosensitizer and TEMP.⁵ Another very common test employs histidine as a chemical trap. Its oxidation results in the formation of a *trans*-annular peroxide, whose reaction with *p*-nitrosodimethylaniline (RNO) is followed by UV-vis absorption spectrophotometry (methodology C).⁶ Alternatively, photophysical experiments combined with histidine degradation followed by HPLC can be used to determine reaction rate constants (methodology C').⁷ Consumption of 1,3-cyclohexadiene-1,4-diethanoate through specific reaction with $^1\text{O}_2$,

Table 14.1. Quantum yield of singlet oxygen formation and quenching constant of $^1\text{O}_2$ by drugs.

Entry	Family	Drug	Φ_{Δ}	$k_q (^1\text{O}_2)/\text{M}^{-1} \text{s}^{-1}$	Solvent ^a	Method ^b	References
1	NSAID	Benoxaprofen	0.4/0.21		np	(A)	11 and 12
			0.18/<0.01 ^c		p	(A)	12 and 13
2		Carprofen	0.32		np	(A)	12 and 14
			<0.01 ^c		p	(A)	12
3		Carprofen photoproduct	0.18		np	(A)	14
4		Naproxen	0.27		np	(A)	12
			0.25 ^c		p	(A)	12
5		Tiaprofenic acid	0.62		np	(A)	12
			0.22 ^c		p	(A)	12
6		Tiaprofenic acid photoproduct	0.57		np	(A)	12
7		Suprofen	0.69		np	(A)	12
			0.37 ^c		p	(A)	12
8		Suprofen photoproduct	0.62		np	(A)	12
9		Ketoprofen	0.39		np	(A)	12
			<0.01 ^c		p	(A)	12
10		Ketoprofen photoproduct	0.29		np	(A)	12
11		Nabumetone	0.19		np	(A)	15
12		Piroxicam	<0.01 ^d		np	(A)	16 and 17
			<0.01		p	(A)	17
13		Piroxicam metabolite	0.35 ^d		np	(A)	17
			0.18		p	(A)	17
14	Quinolone	Norfloxacin		11.8×10^6	np	(A)	18
			0.065/0.081 ^e	1.8×10^6	p	(A)	18–20
15		Pefloxacin	0.045 ^e	12×10^6	p	(A)	20
16		Ciprofloxacin	0.092 ^e	5.2×10^6	p	(A)	18 and 20
17		Ofloxacin	0.13 ^f /0.076 ^e	5.6×10^6	p	(A')/(A)	20 and 21
18		Lomefloxacin	0.072 ^e	18×10^6	p	(A)	20
19		Enoxacin	0.061 ^e	1.4×10^6	p	(A)	20
20		Rufloxacin	0.32 ^g		p	(A)	22
21		Fleroxacin	0.029 ^e	6.9×10^6	p	(A)	20
22		Fleroxacin <i>N</i> -oxide metabolite	0.028	3.6×10^6	p	(A)	20
23		<i>N</i> -Demethylfleroxacin metabolite	0.060	19×10^6	p	(A)	20

24		Cinoxacin	0.16 ^e	1.8×10^6	p	(A)	20
25		Flumequine	0.34 ^e	3.4×10^6	p	(A)	20
26		Nalidixic acid	0.15	25×10^7	p	(A)	23
27		Pipemidic acid	0.016	4.6×10^6	p	(A)	20
28		Piromidic acid	0.015	6.1×10^6	p	(A)	20
29	Statin	Atorvastatin photoproduct	0.3 ⁱ	1.5×10^{8h}	np		24
				4.0×10^{8h}	p	(A) and (B)	24 and 25
30		Atorvastatin metabolite		4.7×10^{8j}	np		24
				2.9×10^{8j}	p		24
31		Rosuvastatin photoproduct	0.3		np	(A)	26
32	Phenothiazine	Perphenazine	0.20		np	(A)	27
33		Fluphenazine hydrochloride	0.20		np	(A)	27
34		Thioridazine hydrochloride	0.23		np	(A)	27
35		Levomepromazine	0.36		p	(C)	28
36		Cyamemazine photoproduct	0.45		p	(C')	7
37		Chlorpromazine hydrochloride	0.27 ^d	6.1×10^6	np	(A)	16 and 29
		Chlorpromazine	Below detection	1.7×10^7	p	(A)	29
38		Mequitazine	0.28 ^d		np	(A)	16
39	Miscellanea	Furosemide	0.047	5.2×10^7	np	(A)	30
			0.12		p	(D)	10
40		Bumetanide	0.07		p	(C)	31
41		Quinine	0.36	1.4×10^7	p	(A')	32
42		Mefloquine	0.38	5.4×10^6	p	(A')	32
43		Quinacrine ^k	0.013	4.6×10^7	p	(A')	32
44		Amodiquine	0.011	4.4×10^7	p	(A')	32
45		Primaquine	<0.005	2.6×10^8	p	(A')	32
46		Chloroquine	<0.005	5.9×10^6	p	(A')	32
47		Hydroxychloroquine	<0.005	2.3×10^7	p	(A')	32
48		Lumidoxycycline	$\leq 0.02^l$		p	(A)	33
49		Doxycycline photoproduct	0.05 ^l		p	(A)	33
50		Adriamycin	0.02		p	(A)	34
51		Daunomycin	0.02		p	(A)	34
52		Daunomycinone	0.03		p	(A)	34
53		Nimodipine	0.085 ^d	3.4×10^5	np	(A)	35
54		Felodipine	0.003 ^d	8.6×10^5	np	(A)	35

(continued)

Table 14.1. (continued)

Entry	Family	Drug	Φ_{Δ}	$k_q(^1\text{O}_2)/\text{M}^{-1}\text{s}^{-1}$	Solvent ^a	Method ^b	References
55		Gilvocarcin V	0.039 ^m		p	(B)	36 and 37
56		Gilvocarcin M	0.031 ^m		p	(B)	36
57		Naphazoline	0.2		np	(A)	38
58		Lamotrigine	0.11		np	(A')	39
			0.01		p	(A')	39
59		Afloqualone	0.14 ^d		np	(A)	16
60		Thiocolchicoside	0.23		p	(E)	40
61		6-Thioguanine	0.56		p	(A)	41
62		6-Thioguanosine	0.55		p	(A)	41
63		Cinacalcet	0.35		np	(A)	42
			0.35		p	(A)	42

^aIf not specified Φ_{Δ} and k_q are reported in acetonitrile as nonprotic solvent (np) or water as protic solvent (p).

^bMethodology used: $^1\text{O}_2$ emission followed by time-resolved (A) and steady-state (A') near-infrared spectroscopy, formation of TEMPO detected by EPR (B), histidine oxidation determined by UV-vis absorption (C) or HPLC (C'), 1,3-cyclohexadiene-1,4-diethanoate oxidation (D) or dimethylfuran oxidation (E).

^cpD 7.6, drug as carboxylate anion.

^dBenzene.

^eIn neutral phosphate buffer 0.05 M in D_2O .

^fIn neutral phosphate buffer 0.01 M in D_2O .

^gIn neutral phosphate buffer 0.01 M in 90/10 $\text{D}_2\text{O}/\text{H}_2\text{O}$.

^hQuenching by atorvastatin.

ⁱMethanol.

^jQuenching by atorvastatin metabolite.

^k $^1\text{O}_2$ formation associated with photoproducts formation.

^lIn acetic acid.

^mIn 45/55 DMSO/ H_2O mixture.

monitored by HPLC, corresponds to methodology D.⁸ Finally, an analytical technique has also been employed based on 2,5-dimethylfuran, which reacts with $^1\text{O}_2$ to give a mixture of products, analyzed by gas chromatography coupled to mass spectrometry (methodology E).^{9,10}

14.2. Singlet Oxygen Formation by Nonsteroidal Anti-Inflammatory Drugs

Nonsteroidal anti-inflammatory drugs are widely used for the treatment of pain. Among them 2-arylpropionic acids (APAs) are well known for their ability to induce skin photosensitivity at clinical level.¹ Involvement of a Type-II mechanism in the photosensitization by some of these drugs has been suggested on the basis of indirect *in vitro* assays like photohemolysis.⁴³ Moreover, benoxaprofen (BXP), naproxen (NP), tiaprofenic acid (TPA), suprofen (SUP), ketoprofen (KP) and carprofen (CP) have been reported to induce singlet oxygen formation with Φ_Δ ranging from *ca.* 0.2 to 0.7 in acetonitrile (Figure 14.1, Table 14.1, entries 1–10).^{11–14} However, these quantum yields exhibit a marked solvent dependence (Table 14.2) due to the occurrence of competing photochemical reactions such as photodecarboxylation, photodehalogenation or photoreduction.¹² The most important factor corresponds to the pH of the medium. In aqueous buffered solution, the Φ_Δ values are negligible for BXP, KP and CP, whereas they suffer a large decrease for TPA and SUP. By contrast, no significant changes are observed for NP. This tendency is also found for mixtures of acetonitrile/ethanol (4/1) basified with KOH, with the exception of CP (Table 14.2).

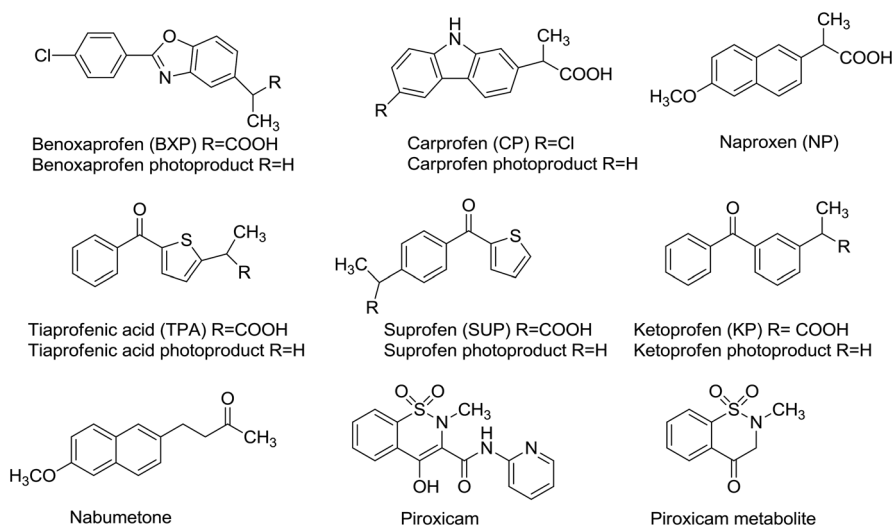


Figure 14.1. Structures of the considered NSAIDs (parent drugs, photoproducts or metabolite).

Table 14.2. Solvent dependence of $^1\text{O}_2$ production by NSAIDs.

Drug	Φ_{Δ}	
	MeCN/EtOH	MeCN/EtOH/KOH
NP	0.26	0.29
BXP	0.14	0.05
KP	0.20	<0.01
TPA	0.38	0.20
SUP	0.63	0.37
CP	0.28	0.21

Indeed, the solvent dependence of $^1\text{O}_2$ sensitization by APAs is linked to their photoreactivity. Naproxen, which is relatively photostable with a photodecomposition quantum yield Φ_{dec} of only 0.012,⁴³ does not show Φ_{Δ} changes in the considered media. A different photobehavior is observed for the BXP, KP, TPA and SUP, whose quantum yields are highly affected by the pH of the solution. These four compounds suffer efficient photodecarboxylation (Φ_{dec} *ca.* 0.18 and 0.75 for BXP and KP, respectively)^{43,44} giving rise to the photoactive products shown in Figure 14.1. The key step in this process is an intramolecular electron transfer from the deprotonated carboxylic acid to the excited chromophore. Additional photoreactivity channels operate in KP and TPA, which possess a benzophenone-like chromophore and undergo photoreduction of the excited carbonyl in the presence of good H-donors such as ethanol (see Table 14.2). Indeed, this effect is more pronounced for KP because its lowest triplet excited state is $n\pi^*$ in nature. The 2-benzoylthiophene moiety of TPA exhibits an almost degenerated $n\pi^*/\pi\pi^*$ state, reducing the H-abstraction capability. Finally, CP is photolabile both in organic and aqueous media. In the former medium, the main process is dehalogenation (Φ_{dec} *ca.* 0.32) that leads to carprofen photoproduct shown in Figure 14.1. The efficiency of this process is not sensitive to pH. In phosphate buffer solution, extensive polymerization takes place.^{14,43} Additional studies have been performed in water-in-oil and oil-in-water microemulsions based on anionic and cationic surfactants, where the APAs properties are also highly affected by the medium; the Φ_{Δ} are higher in microheterogeneous systems than in bulk aqueous solution, with the exception of KP that shows in general very low values.⁴⁵ Location of the drugs in the interfacial region of the microemulsions renders them good candidates for Type-II damage in cell membranes.

In this context, the importance of the environment has also been investigated by means of model systems including covalently linked biological components. A clear example relies on the photodynamic oxidation of cholesterol (Ch), which has been addressed by means of KP- or TPA-based dyads (KP- α/β -Ch and TPA- α/β -Ch, respectively).⁴⁶ Direct Φ_{Δ} measurements have shown that generation of $^1\text{O}_2$ is almost negligible for KP- α -Ch, whereas a value of *ca.* 0.5 has been determined for TPA- α -Ch. In the case of the β -isomers, no significant changes are observed for TPA, whereas for KP Φ_{Δ} it increases to 0.2, a value close to that determined for the free drug in acetonitrile. The

diverging behavior of the KP-derived dyads is based on the fast intramolecular hydrogen abstraction taking place only in the case of the α -isomer. Thus, this dyad is an appropriate model for a clean Type-I mechanism, while the TPA derivatives are suitable systems for Type-II photosensitization. This example clearly illustrates the difficulties to anticipate the Type-I/II reactivity of a drug as it is essential to consider the electronic configuration of the photosensitizer together with the nature of the environment.

Other NSAIDs different from APAs have also been reported as $^1\text{O}_2$ sensitizers (Table 14.1, entries 11–13). Nabumetone is a prodrug that acts through its active metabolite, namely the 6-methoxy-2-naphthylacetic acid. Photo-physical properties of nabumetone are dominated by its 2-methoxynaphthalene chromophore, also present in NP.¹⁵ Piroxicam is an interesting example because the observed phototoxicity has been attributed to one of its metabolites, 2-methyl-4-oxo-2*H*-1,2-benzothiazine-1,1-dioxide (Figure 14.1), rather than to the parent drug itself.¹⁷ Accordingly, *in vitro* assays performed on mononuclear human cells point to the occurrence of a Type-II process mediated by the metabolite. The Φ_{Δ} values, obtained both in protic and nonprotic media, are in agreement with this hypothesis as $^1\text{O}_2$ formation is hardly detected for piroxicam, while efficiencies in the range of 0.2–0.35 are determined for the metabolite.^{16,17}

14.3. Singlet Oxygen Formation by Quinolone Antibacterial Agents

Quinolones are synthetic broad spectrum antibacterial agents. The first generation of these drugs began with nonhalogenated derivatives, such as nalidixic acid or oxolinic acid; however, nowadays the fluoroquinolones (FQs) bearing a fluorine atom in position 6 and/or 8 are dominating the market (Figure 14.2). In spite of their advantageous pharmacological properties, the quinolones belong, together with NSAIDs, to the main groups of drugs that induce phototoxicity as a significant side effect.¹ As a general remark, the reported Φ_{Δ} of FQs are low with values <0.1 , with the exception of rifloxacin and flumequine (Table 14.1, entries 14–25).^{18–21,47} The photochemistry of FQs is governed by numerous factors. One of them concerns their acid–base equilibria, due to the presence of multiple proton binding sites *i.e.* the carboxylate group and the piperiziny ring. At neutral pH, the zwitterionic form predominates. This pH dependence has been studied in detail for norfloxacin, whose Φ_{Δ} shows a maximum at pH 8–9.¹⁸ This result can be explained in part through the pH-dependent quenching of the photogenerated $^1\text{O}_2$ by the FQ with a rate constant that decreases from 7.9 to $1.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ between pD 4 and 7.5, while it increases about 20-fold in alkaline solution.¹⁸ For the zwitterionic form of norfloxacin Φ_{Δ} is also sensitive to complexation with metal cations as Ca^+ and Mg^{2+} .¹⁹ Another important property for most FQs relies on the efficient triplet excited-state quenching by phosphate ions.^{47,48} This might explain why, in spite of intersystem crossing quantum yields Φ_{ISC}

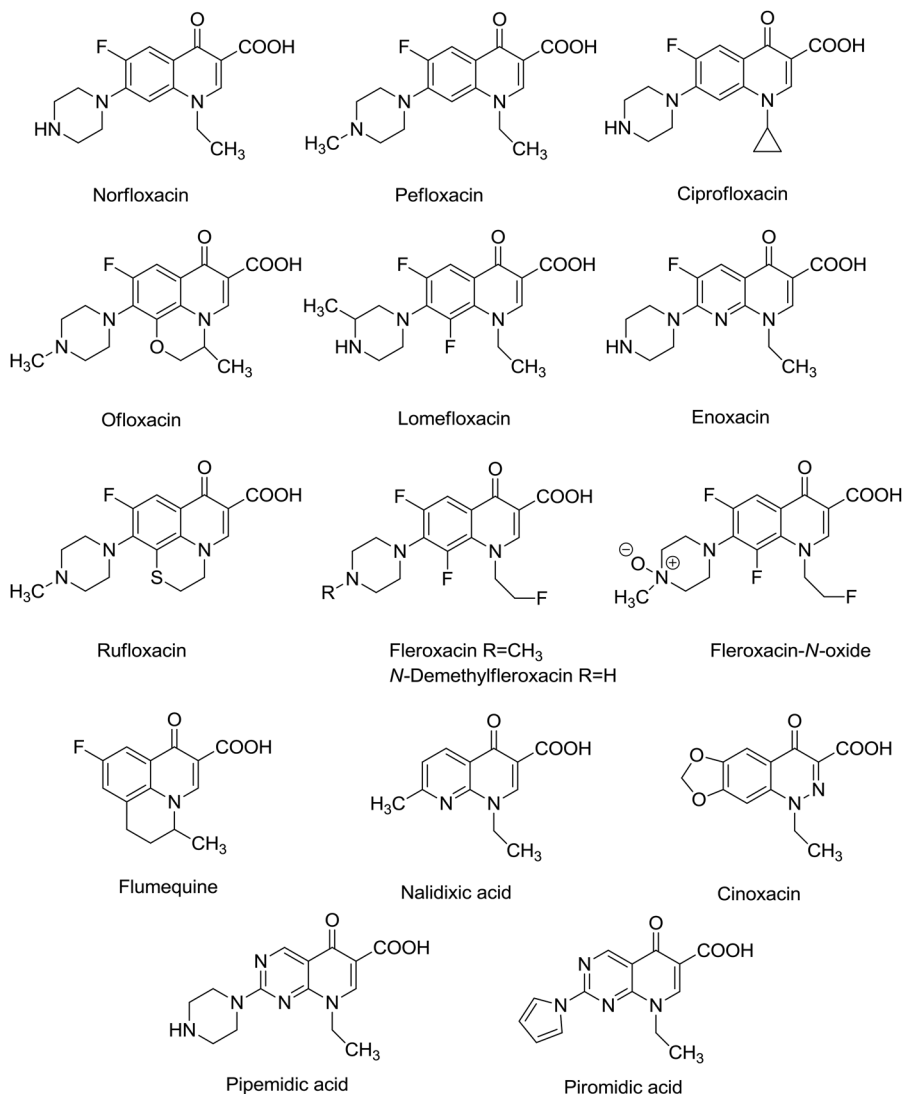


Figure 14.2. Structures of the reported quinolones.

higher than 0.4, most FQs have low Φ_{Δ} in phosphate buffer.⁴⁷ Thus, experimental conditions are critical in the determination of FQ photophysical properties; this can be explained in some cases by the reported diverging data. This is illustrated in Table 14.1 by ofloxacin, whose Φ_{Δ} is divided by two when the concentration of phosphate increases from 0.01 M to 0.05 M.^{20,21} By contrast, rufloxacin is much less influenced by the presence of phosphate ion, and accordingly its Φ_{Δ} (Table 14.1, entry 20) is very close to its Φ_{ISC} of ca. 0.36.^{22,48–50} This has been attributed to the importance of the heteroatom attached to position 8 of the quinolone ring; the sulfur derivative rufloxacin

has a lower triplet excited-state energy than its oxygen analog, ofloxacin.⁵¹ The importance of phosphate quenching is also evidenced in DNA photosensitization experiments.⁴⁸ Although both FQs are able to induce formation of 8-oxo-2'-deoxyguanosine, rifloxacin is 10-fold more efficient in the production of this DNA oxidation marker. In parallel, the Type II/Type I ratio in the oxidation of isolated 2'-deoxyguanosine is higher for the *S*- than for the *O*-substituted FQ.⁴⁸ A singlet oxygen-mediated mechanism is also involved in the photohemolysis and lipid photoperoxidation by rifloxacin.⁵²

The dihalogenated lomefloxacin and fleroxacin are particularly harmful and have been demonstrated to photoinduce tumors on mice.²⁰ However, their Φ_{Δ} are less than 0.1 (Table 14.1, entries 18 and 21). The role of metabolism in $^1\text{O}_2$ photogeneration has been demonstrated to play a minor role for fleroxacin because Φ_{Δ} of its two main metabolites, namely fleroxacin-*N*-oxide and *N*-demethylfloxacin, is not significantly higher than that of the parent drug (Figure 14.2, Table 14.1, entries 22 and 23).²⁰

As regards nonhalogenated quinolone, such as nalidixic acid and cinoxacin, a Φ_{Δ} of around 0.15 has been determined, while for pipemidic acid and piromidic acid the reported values are <0.1 (Table 14.1).^{20,23}

14.4. Singlet Oxygen Formation: The Case of Statin Drugs

Statin drugs are prescribed worldwide for the treatment of cholesterol. Clinical cases of cutaneous reactions have been reported and associated with photosensitivity disorders. However, no evidence has been provided to label statin drugs as intrinsic photosensitizers. Photochemical and photobiological studies have demonstrated that, indeed, sensitivity might be mediated by the statin photoproducts.^{24–26,53} The first reported example corresponds to atorvastatin.²⁵ A detailed analysis of its photoproducts has revealed that the phenanthrene-like derivative displays the properties of a good Type-II sensitizer (Figure 14.3). Singlet oxygen, observed by both the TEMPO/EPR methodology and the direct time-resolved near-infrared spectroscopy, is photogenerated with a quantum yield of *ca.* 0.3 (Table 14.1, entry 29). The photoproduct capability to oxidize proteins has been demonstrated by studying the degradation of tryptophan. Moreover, this reactive oxygen species is able to react with the parent drug with a rate constant k_q of $1.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$.²⁵ A similar k_q has also been reported for the reaction of $^1\text{O}_2$ with an atorvastatin *ortho*-hydroxy metabolite (Figure 14.3).²⁴ In the case of rosuvastatin, the photoactive compound is the primary dihydrophenanthrene-like product resulting from photochemical electrocyclization.²⁶

14.5. Singlet Oxygen Formation by Phenothiazine Drugs

Phenothiazines are neuroleptic drugs generally prescribed for their tranquilizing and antipsychotic properties. Chlorpromazine has been used as a prototype for this class of drugs, and since its introduction for treatment of mental disorders, a large number of structural analogs have been developed.

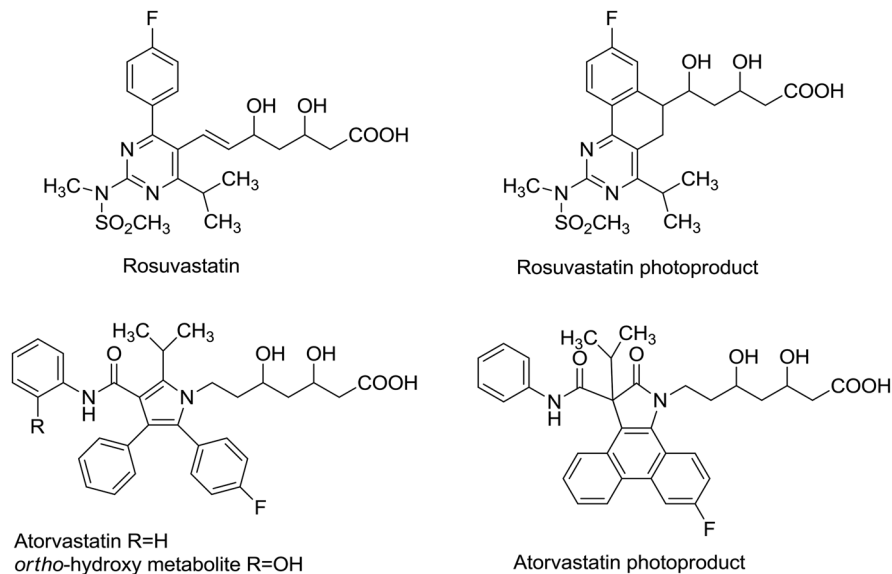


Figure 14.3. Structures of atorvastatin, rosuvastatin, their photoproducts and a metabolite.

All these compounds share the same phenothiazine chromophore substituted at C2 and bearing a side chain at position N-10 (Figure 14.4).

Singlet oxygen quantum yields ranging from 0.2 to 0.36 have been reported for phenothiazines (Table 14.1, entries 32–38).^{7,16,27–29} Chlorpromazine photogenerates $^1\text{O}_2$ only in organic solvents,^{16,29} whereas in D_2O fast de-excitation of the triplet state occurs through a competing photodegradation pathway.²⁹ In both media, the drug reacts with $^1\text{O}_2$ with quenching rate constants of 10^6 – $10^7 \text{ M}^{-1} \text{ s}^{-1}$. Interestingly, the Φ_Δ value in Triton X-100 micelles is twice than in benzene,²⁹ thus suggesting that $^1\text{O}_2$ formation can occur in membranes. Further experiments demonstrate that chlorpromazine is able to photoinduce oxidation of phospholipids and cholesterol in liposome membranes mainly by a Type-II process.²⁹ Perphenazine also exhibits this type of photoreactivity, which is less pronounced for others members of the family like fluphenazine, thioridazine and levomepromazine.^{27,28} In the case of cyamemazine, the determined Φ_Δ has not been ascribed to the parent drug but to its main photoproduct (Table 14.1, entry 36), oxidized both at the sulfur of the phenothiazine ring and at the nitrogen of the side chain.⁷

14.6. Photogeneration of Singlet Oxygen by Drugs: Miscellaneous

This section includes the data available for drugs of different structures and therapeutic use (Figure 14.5, Table 14.1, entries 39–63). Furosemide and bumetanide are diuretic agents with low Φ_Δ .^{10,30,31} Among the antimalarial

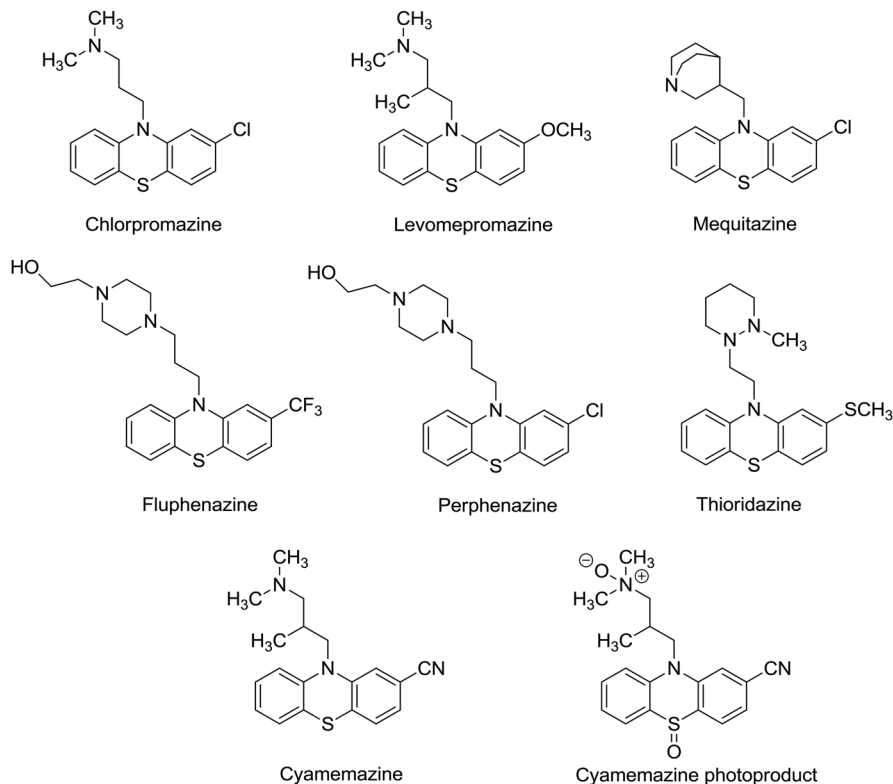


Figure 14.4. Structures of phenothiazine compounds.

drugs quinine, mefloquine, quinacrine, amodiaquine, primaquine, chloroquine and hydroxychloroquine only the first two compounds are able to photosensitize $^1\text{O}_2$ in good yield (Figure 14.5, Table 14.1, entries 41–47).³² Accordingly, a Type-II process is proposed for the cellular phototoxicity revealed by MTT test on fibroblasts as well as for tryptophan and histidine oxidation.⁵⁴ In the case of quinacrine, the obtained Φ_Δ has been associated with photoproduct formation.³²

The tetracycline antibiotics, the antihypertensive agents nimodipine or felodipine, and the gilyvocarcin antitumor drugs exhibit very low Φ_Δ of the order of 10^{-2} (Figure 14.5, Table 14.1, entries 53–56).^{33–37} By contrast, the vasoregulator drug naphazoline, the antiepileptic lamotrigine, and the muscle-relaxant agents aflaqualone and thiocolchicoside are better $^1\text{O}_2$ photosensitizers.^{16,38–40} In this context, a Type-II process has been proposed to be at the origin of lipid peroxidation mediated by lamotrigine and thiocolchicoside.^{39,40} The thiopurine drugs used as cancer therapeutic and immunosuppressive agents show high Φ_Δ (Table 14.1, entries 61 and 62); this agrees with their photoactivity toward DNA and the increased risk of skin cancer in treated patients.^{41,55,56} Finally, the calcimimetic agent cinacalcet is an interesting

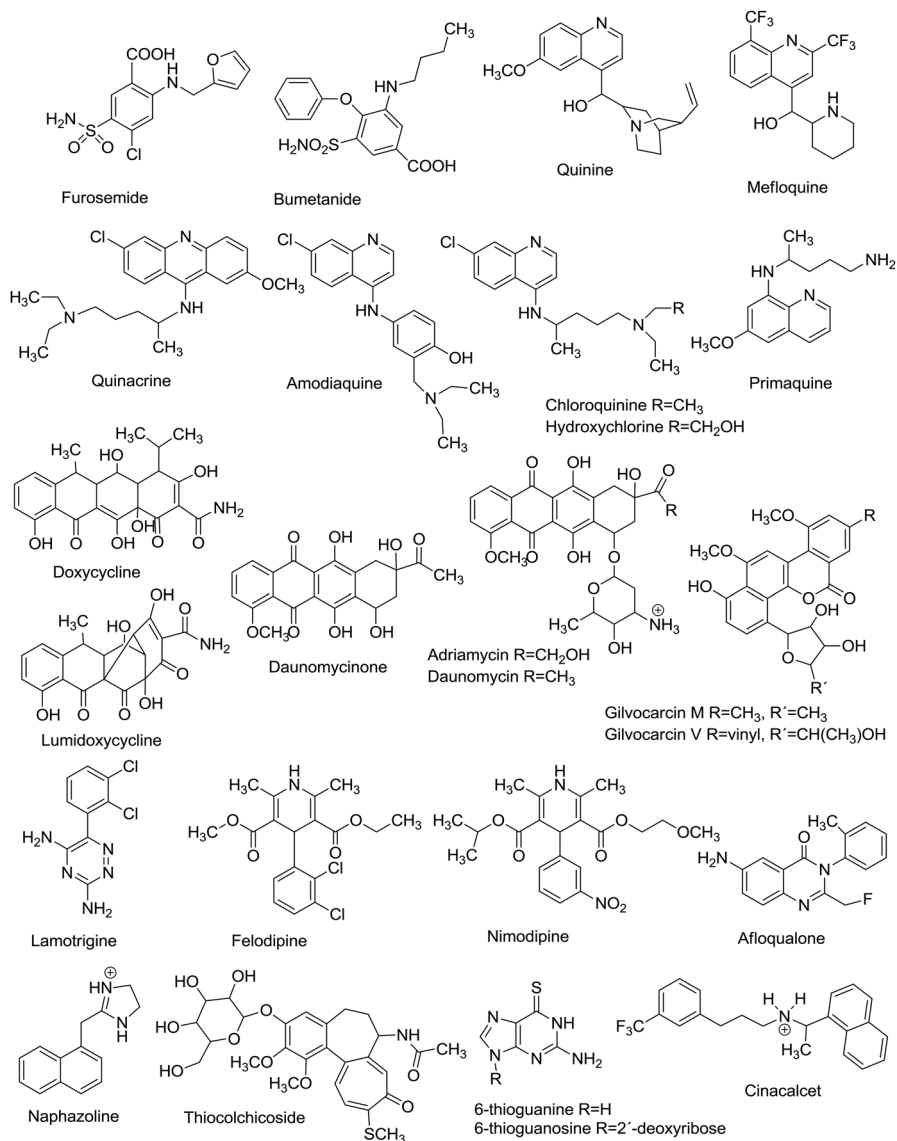


Figure 14.5. Structures of other drugs photosensitizing $^1\text{O}_2$ formation.

case.⁴² This drug contains the naphthalene chromophore and consequently photoinduces $^1\text{O}_2$ formation in good yield (Table 14.1, entry 63). Binding of cinacalcet to human serum albumin does not result in a significant change of its triplet excited-state quantum yield; however, it is remarkable that the Φ_Δ in the protein environment is negligible due to inefficient quenching by oxygen in the binding sites.⁴²

14.7. Summary and Outlook

Photosensitized generation of singlet oxygen has been reported for a number of therapeutic agents, belonging to different structural families. With the exception of drugs employed for photodynamic therapy (not included in the present chapter), whose mode of action is usually based on Type-II photo-oxidation mechanism, photosensitized production of singlet oxygen is associated with photosensitivity side effects. In this context, although the involvement of this reactive species has been suggested in many cases on the basis of indirect chemical or biological observations, quantitative data supported by accurate measurements have been obtained for a relatively reduced number of drugs. The most reliable information has been collected and discussed in the present chapter, which is not intended to provide a comprehensive compilation but rather a general overview of the topic. From the critical examination of the existing data, it becomes clear that singlet oxygen may indeed mediate photosensitized oxidation of biomolecules by some therapeutic drugs, although this process seems to be efficient ($\Phi_{\Delta} > 0.2$) in a relatively limited number of cases. In any case, it would be convenient to include evaluation of singlet oxygen formation in photosafety testing, as part of the toxicological assessment of new drug candidates possessing photoactive chromophores, before their introduction in the market.

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Chapter 15

Nanofibers and Nanocomposite Films for Singlet Oxygen-Based Applications

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15.1. Nanomaterials – Unique Sources of Singlet Oxygen

Design of molecular systems and materials with rapid, specific responses to light stimuli is a focus of various fields of medicine and materials science. The activation of a specific function *via* light has emerged as an easy and rapid on/off switching mechanism for controlling the reactivity. These attributes have stimulated the development of novel constructs with singlet oxygen-producing, gas-releasing, sensing, energy-converting, photochromic, or photocatalytic properties.^{1–3} It is worth mentioning that recent interest in singlet oxygen-mediated processes is motivated by progress made in spectroscopic methods,⁴ in tracing of singlet oxygen fate in cells,⁵ in the mechanisms of controlling singlet oxygen production,⁶ in singlet oxygen reactivity,⁷ and in the formulation of novel (nano)materials capable of photosensitizing singlet oxygen.⁸ In addition, the rise in bacterial drug resistance has spurred a great deal of recent research focused on the development of alternative anti-infective strategies, which includes singlet oxygen-producing materials.⁹ The mechanism by which $^1\text{O}_2$ attacks micro-organisms is nonsite specific, and as such, the development of bacterial resistance to it is unlikely.

Singlet oxygen, the excited electronic state of molecular oxygen, is involved in numerous oxidative processes that cause serious damage to biological materials. Its oxidative potential can be exploited in chemical syntheses, photodegradation of pollutants, photodynamic treatment of cancer, and antimicrobial photodynamic therapy. Although $^1\text{O}_2$ can be generated in a number of ways, photosensitized production is the most common method.^{10,11} Many reported PSs have several drawbacks, including poor water solubility, aggregation, toxicity, and instability. Therefore, in addition to common organic or inorganic compounds that have been used as PSs,^{10,12} the photosensitizing activities of materials with immobilized PSs have also been investigated.¹³ These materials can be prepared by immobilization of PSs in suitable organic or inorganic matrices, adsorption onto surfaces, intercalation into layered materials, or encapsulation in materials with sufficient oxygen permeability.^{7,13–15} The advantage of immobilizing PSs is that it provides a homogeneous environment around the PS molecules, makes them easy to separate from the reaction mixture, and can increase their photostability. It is worth noting that in the very first experimental proof of $^1\text{O}_2$, the PS used was immobilized on a solid support.¹⁶

Singlet oxygen has a short lifetime that limits its reactivity to the proximity of the site where the $^1\text{O}_2$ was formed. The root-mean-square distance d that $^1\text{O}_2$ would move in a time period t can be expressed by the following equation:

$$d = (6tD)^{1/2}, \quad (15.1)$$

where D is the relevant diffusion coefficient for oxygen. For example, in water with a D of $2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, the distance d is 355 nm at the time at which 5% of the originally produced $^1\text{O}_2$ remains (*i.e.*, $t = 3 \times \tau_\Delta$, where τ_Δ

is the $^1\text{O}_2$ lifetime).¹⁷ Singlet oxygen photosensitized by PSs confined in materials can be formed at the surface and/or in the bulk of the material, and so the reactions of $^1\text{O}_2$ with a target species, commonly located at the surface or in the close surroundings, depend on by the ability of $^1\text{O}_2$ to reach these locations. According to eqn (15.1), the effective distance is dictated by the material type. For example, in polystyrene with a D of approximately $3 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ and a $^1\text{O}_2$ lifetime of $20 \mu\text{s}$, only $^1\text{O}_2$ formed within approximately a 100 nm polymer layer can reach the surface to cause reactions. This example highlights the limited utility of encapsulated PSs in bulk materials. In contrast, nanomaterials with dimensions of approximately 100 nm and less enhance the effectiveness of $^1\text{O}_2$ -based photoreactions provided that the material itself does not quench $^1\text{O}_2$. A wide variety of nanomaterials such as quantum dots, silicon nanocrystals, polymer nanoparticles, liposomes, and carbon nanotubes have been investigated as potential carriers of PSs in photodynamic therapy.^{8,18,19} Among the many nanomaterials that produce $^1\text{O}_2$, polymer nanofibers and nanocomposite films have emerged as potential multifunctional platforms with antibacterial, antiviral, and oxidative properties. The properties, functions, and applications of these nanomaterials are surveyed in this chapter.

15.2. Nanofiber Materials

Polymer nanofibers have quickly become widely used nanomaterials in many applications. These nanomaterials have several advantages over bulk polymers, as they possess large specific surface areas, high porosity, mechanical flexibility, and light transparency and are light weight and low cost; nanofibers also have small diameters ranging from tens to hundreds of nanometers (Figure 15.1(A)).²⁰ During the past two decades, nanofibers have increasingly been used as filtration materials,²¹ scaffolds for the regeneration of bone, skin, heart, and blood vessels,^{22–24} carriers for catalytic reactions,²⁵ optical sensors,²⁶ smart textiles,²⁷ and multifunctional devices.²⁸ Although many techniques have been developed to fabricate polymer nanofibers,²⁴ the most common method is by electrospinning from a polymer solution.^{20,29,30} Electrospinning is an easy and reproducible way to synthesize robust nanofiber materials in the form of membranes or textiles and does not require advanced methods for the product separation from a reaction mixture, as is required for most nanoparticles.

The photosensitized production of $^1\text{O}_2$ can be accomplished by integrated PSs with the nanofibers. There are two basic strategies leading to such materials: (a) prefuctionalization by mixing PSs or PSs covalently attached to polymer chains with polymer solutions and using these directly for electrospinning (see Section 15.2.1) or (b) postprocessing, in which the PS is bound to the functionalized surface of the electrospun nanofibers *via* noncovalent or covalent bondings interactions (see Section 15.2.2).

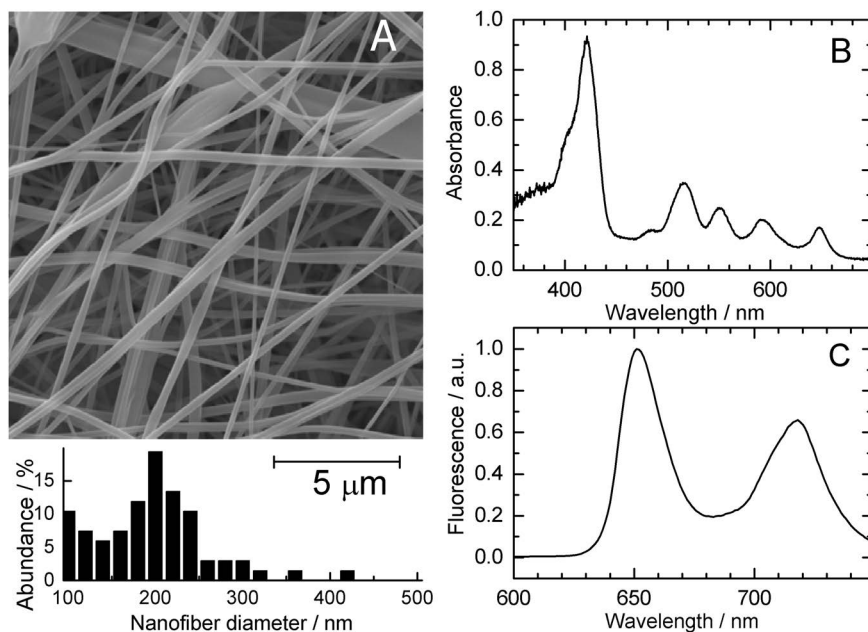


Figure 15.1. Polystyrene nanofiber material with encapsulated TPP (1 wt%). (A) SEM image with the corresponding diameter distribution; (B) UV/Vis absorption and (C) fluorescence emission spectra. Adapted with permission from ref. 32 and 33. Copyright 2010 and 2014, American Chemical Society.

15.2.1. Encapsulated Photosensitizers in Polymer Nanofibers

15.2.1.1. Preparation and Properties. Electrospinning of polymer solutions containing dissolved 5,10,15,20-tetraphenylporphyrin (TPP) produced membranes with TPP encapsulated in nanofibers (Figure 15.1(A)).^{31–35} Encapsulated TPP imparts a unique photofunction to the nanofibers because TPP is an efficient PS of $^1\text{O}_2$ in solutions. The diameter of the nanofibers (100–400 nm) is comparable to the oxygen diffusion radius (eqn (15.1)) in polymers in which oxygen has high diffusion coefficients (*e.g.*, polystyrene, polyurethane).³⁵ The absorption (Figure 15.1(B)) and fluorescence emission (Figure 15.1(C)) spectra of encapsulated TPP were similar to those of TPP in CH_2Cl_2 , indicating that TPP remained monomeric in polymer matrices and the tendency to form porphyrin aggregates was low. Such aggregation would be undesirable because it causes significant quenching of excited states, including triplet states, and thus causes a considerable decrease in sensitizing activity. The fluorescence properties of the nanofibers allowed for investigation of the nanofiber morphology by utilizing fluorescence lifetime imaging microscopy (Figure 15.2(A)).³²

The quenching of the triplet states of encapsulated TPP by oxygen (Figure 15.3(A)) led to the formation of $^1\text{O}_2$ with a rate constant of $2.5 \text{ s}^{-1} \text{ Pa}^{-1}$ (Figure 15.3(B)). The lifetimes of the triplet states were not affected by

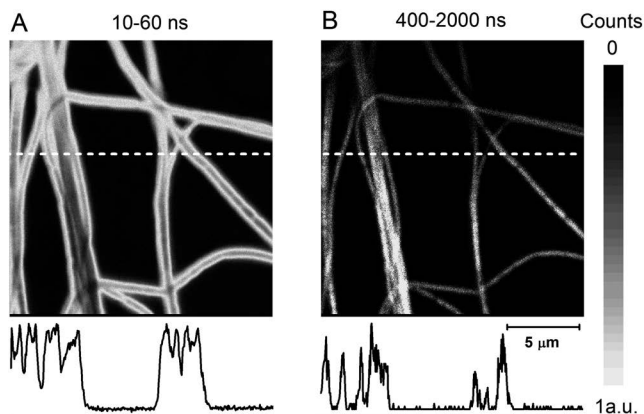


Figure 15.2. Fluorescence lifetime imaging spectroscopy of polystyrene nanofiber material containing encapsulated TPP. (A) Fluorescence intensity image; (B) SODF intensity image collected 400–2000 ns after excitation. The corresponding fluorescence intensity profiles along the dotted line are at the bottom. Reprinted with permission from ref. 32. Copyright 2010, American Chemical Society.

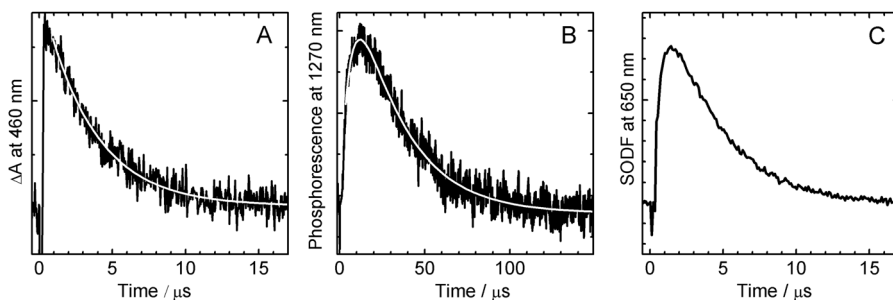


Figure 15.3. Polyurethane nanofiber material containing encapsulated TPP (1 wt%) in 101 kPa oxygen. (A) Kinetics of the triplet states recorded at 460 nm; (B) production of $^1\text{O}_2$ recorded by its phosphorescence at 1270 nm. The concentration of $^1\text{O}_2$ at time t is governed by the kinetics of the $^3\text{PS}^*$ states and by the reactivity of $^1\text{O}_2$ in a given environment, as expressed by the following equation: $[\text{}^1\text{O}_2]_t = A\tau_\Delta/(\tau_T - \tau_\Delta)(\exp(-t/\tau_T) - \exp(-t/\tau_\Delta))$, where A is a parameter, τ_T is the lifetime of the triplet states responsible for the $^1\text{O}_2$ formation, and τ_Δ is the $^1\text{O}_2$ lifetime. (C) SODF intensity recorded at the maximum of the fluorescence band. The solid lines in (A) and (B) are least-squares exponential fits. Adapted with permission from ref. 38. Copyright 2010, American Chemical Society.

the surrounding media (Table 15.1). In contrast, the lifetime of $^1\text{O}_2$ in pure water, that is $3.5 \mu\text{s}$,³⁶ was doubled when $^1\text{O}_2$ was produced by nanofibers immersed in water and decreased upon the addition of NaN_3 , an effective $^1\text{O}_2$ quencher. These experiments clearly indicate the ability of $^1\text{O}_2$ to diffuse to the polymer surface, where $^1\text{O}_2$ molecules interact with the solvent and the components of the solution. Similar results were obtained for encapsulated ZnTPP and zinc phthalocyanine photosensitizers.³⁷ These nanofiber

Table 15.1. Lifetimes of the porphyrin triplet states (τ_T) and of $^1\text{O}_2$ (τ_Δ) produced by encapsulated TPP in polyurethane nanofibers in an air atmosphere or immersed in air-saturated solutions.³¹

Surroundings	$\tau_T/\mu\text{s}$	$\tau_\Delta/\mu\text{s}$	
		Measured	Literature ^a
Air	18	21	— ^b
D ₂ O	18	19	68
H ₂ O	18	6	3.5
H ₂ O, 0.01 M NaN ₃	18, 2 ^c	5	—

^aLifetime of $^1\text{O}_2$ in the given environment.^bRadiative lifetime of unperturbed $^1\text{O}_2$ is 72 min.^cBiexponential decay.

materials efficiently harvested visible light and generated $^1\text{O}_2$ with a lifetime of approximately 15 μs in an air atmosphere. In general, $^1\text{O}_2$ produced by the nanofibers successfully oxidized inorganic or organic compounds in the surrounding solution.^{31,35,37}

The high local population of porphyrin triplet excited states and their interaction with $^1\text{O}_2$ caused the repopulation of the TPP singlet excited states (Figure 15.3(C)). The process is known as singlet oxygen-sensitized delayed fluorescence (SODF).^{32,38} Because the intensity and kinetics of SODF are affected by the local oxygen concentration, SODF is a sensitive indicator of an oxygen presence in a given material. Hence, SODF spectroscopy was proposed as a suitable method for spatially resolved imaging of $^1\text{O}_2$ inside the polymer nanofibers and other materials (Figure 15.2(B)). This technique is limited to materials with immobilized PS triplets that cannot diffuse and come in close contact with one another and are accessible to oxygen.

The selection of a spinnable polymer with sufficient oxygen permeability is crucial for ensuring the efficient transport of oxygen to sites with excited PS molecules.³⁵ The diffusion length of $^1\text{O}_2$ in polymers with high oxygen permeability is typically tens to hundreds of nanometers.³⁸ This high permeability leads to photo-oxidation reactions at the nanofiber surfaces or in close proximity to them. To obtain higher photo-oxidation efficiencies would require the organization of the PSs near the fiber surfaces, which is not easy to control,³⁹ and/or postprocessing of the polymer surface without damage to the nanofiber morphology (see Section 15.2.2). Among the other parameters that can be used to control the formation of $^1\text{O}_2$ is temperature. A decrease in temperature slows the kinetics of the TPP triplet-state quenching by oxygen due to a decrease in the oxygen diffusion coefficient; however, the lower temperature did not significantly influence the yield of $^1\text{O}_2$ (Figure 15.4(A) and (B)).³³ These results indicate that nanofibers are good PSs even at temperatures below 10 °C.

Although the oxidation of species dissolved in water droplets can be achieved using a superhydrophobic material modified by a partially embedded polar PS,⁴⁰ surface hydrophilicity (wettability) itself plays a crucial role in achieving the efficient photo-oxidation of a chemical substrate/biological

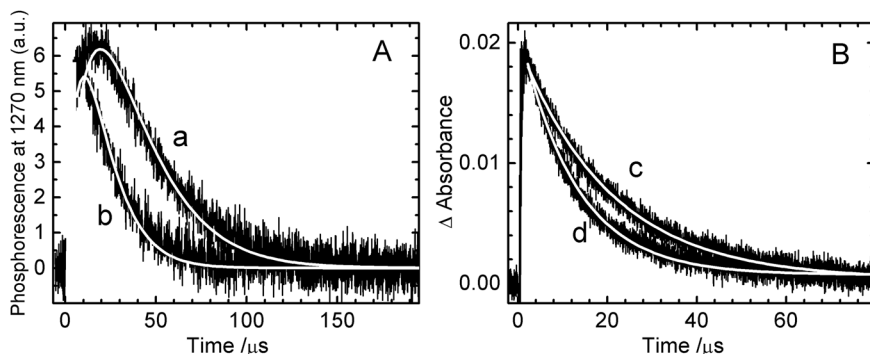


Figure 15.4. Polystyrene nanofiber material with encapsulated TPP in the air atmosphere. (A) Kinetics of the $^1\text{O}_2$ formation and decay at 5 °C (a) and 55 °C (b) fitted using a double-exponential function (see Figure 15.3(B)). (B) Corresponding decay of the TPP triplet states at 5 °C (c) and 55 °C (d) fitted using a single-exponential function (solid lines). Reprinted with permission from ref. 33. Copyright 2014, American Chemical Society.

target by $^1\text{O}_2$.⁴¹ This attribute can be roughly estimated by measuring the apparent contact angles of water droplets (ACA). As an example, postprocessing modifications such as sulfonation, oxygen plasma treatment, and polydopamine coating strongly increased the wettability (ACA $\leq 5^\circ$) of pristine hydrophobic polystyrene nanofibers with encapsulated TPP (original ACA $\approx 130^\circ$) without damaging the nanofiber morphology or causing leakage of PS and without inducing changes in the spectral characteristics of PSs, the lifetimes of the triplet states and $^1\text{O}_2$, or even the oxygen permeability (see Section 15.2.2). The increase in the surface wettability yielded a significant increase in the photo-oxidation rate of dissolved substrates and in the antibacterial activity of the nanofibers (Figure 15.5).

15.2.1.2. Applications. A variety of biocides, including antibiotics, quaternary ammonium salts, triclosan, biguanides, nanoparticles, and chitosan, have been incorporated by various techniques into nanofibers, and they exhibit strong antibacterial activity in standard assays.⁴² The generation of cytotoxic $^1\text{O}_2$ gives rise to the potential for the utilization of nanofiber materials with encapsulated PSs in antimicrobial photodynamic applications. The activity is easier to control by triggering the amount of antibacterial agent, $^1\text{O}_2$, using light intensity and/or the amount of encapsulated PSs. The practically limitless amount of oxygen in air ensures a continuous supply of precursor for the formation of $^1\text{O}_2$.

Recently developed nanofiber materials with encapsulated TPP or zinc phthalocyanine/ZnTPP generated $^1\text{O}_2$ upon irradiation with visible light and efficiently killed both bacteria^{31,33,35,37} and viruses.³⁴ Similarly, polystyrene fibers doped with phthalocyanine complexes exhibited antimicrobial activities under illumination with visible light.⁴³ The antibacterial effect

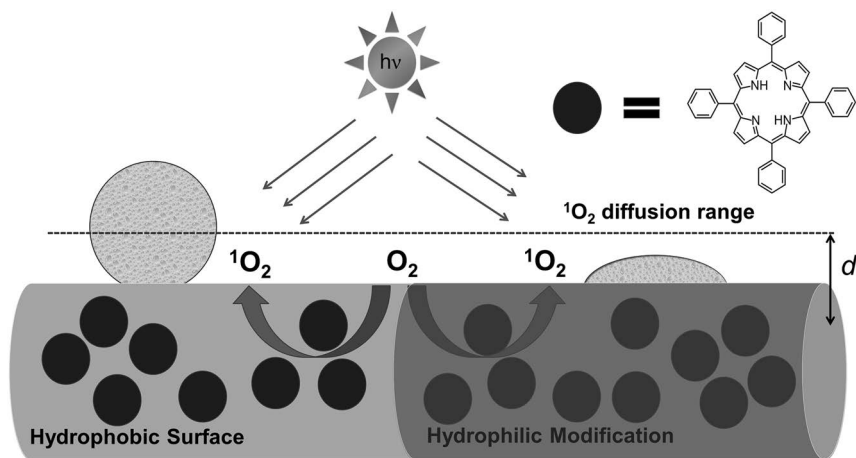


Figure 15.5. Water drop on hydrophobic (left) and hydrophilic (right) surfaces of a nanofiber containing encapsulated TPP compared with the $^1\text{O}_2$ diffusion length (d , see eqn (15.1)). Reprinted with permission from ref. 41. Copyright 2014, American Chemical Society.

decreased at lower temperatures predominantly due to the decrease in oxygen diffusion (Figure 15.4).³³ The selection of the polymer is crucial for biocompatibility and the successful transport of oxygen to excited PS molecules. Because of their transport properties, prepared polyurethane, postprocessed polystyrene, and polycaprolactone nanofiber membranes exhibited strong antibacterial activity and completely inhibited the bacterial growth under visible-light irradiation.^{35,41} In contrast, polyamide 6 nanofiber membranes exhibited lower antibacterial effects due to the low oxygen permeability of the polymer. The low photo-oxidation power of polyamide 6 nanofibers was also confirmed with encapsulated substituted zinc(II) phthalocyanine.⁴⁴ Surprisingly, all the nanofiber membranes exhibited postirradiation antibacterial properties after long irradiation periods due to the minor generation of H_2O_2 .³⁵

Hydrophilic biocompatible polyurethane (Tecophilic) nanofibers containing encapsulated TPP photosensitizer were successfully applied as a wound dressing for the healing of patients with leg ulcers.⁴⁵ These antibacterial materials may be advantageous compared to standard antiseptic treatment options because TPP cannot penetrate the skin or wound and therefore does not interfere with the healing process. Pheophorbide a and its poly-L-lysine conjugate were encapsulated into electrospun polycaprolactone fibers.⁴⁶ These phototriggered materials generated reactive oxygen species including $^1\text{O}_2$ upon irradiation with near-infrared light. Irradiation led to effective killing of cells on the polymer scaffold, allowing the modulation of their proliferation in a controlled fashion. Photoactive micro/nanosized poly(ϵ -caprolactone) fibers were also prepared from the polymer with covalently attached fullerene C_{60} , a good PS for the generation of $^1\text{O}_2$.⁴⁷ These

nanostructured materials were suggested as promising candidates for photodynamic therapy of cancer and treatment of multidrug-resistant pathogens. The strong photoantiviral effects toward nonenveloped polyomaviruses and enveloped baculoviruses were observed on the surfaces of hydrophilic polyurethane and polycaprolactone nanofibers with encapsulated TPP.³⁴

Photosensitizer-doped nanofibers can also be used for the photosensitized degradation of harmful compounds. As a typical example, polyurethane nanofiber materials containing encapsulated TPP were applied for photo-oxidation of 2-chlorophenol⁴⁸ and parabens⁴⁹ in aqueous solutions. Porphyrin TPP harvests blue light; however, absorption at longer wavelengths is rather low. To improve its absorption range, phthalocyanines were also introduced. Polyurethane nanofiber material containing encapsulated zinc phthalocyanine readily photo-oxidized inorganic and organic substrates and also displayed antibacterial effects on their surfaces; however, this PS was less photostable than TPP.³⁷ The polymeric fibers encapsulating lutetium phthalocyanine were found to be promising materials for the photoconversion of 4-nitrophenol.⁵⁰ Zinc phthalocyanine electrospun into polyamide-6 nanofibers was used for the photodegradation of dye Orange G.⁵¹

The potential danger of chemical-warfare attacks led to an increased need for developing novel decontamination methods for the surfaces that could be exposed to chemical-warfare agents. It was found that encapsulated zinc octaphenoxypthalocyanine that produces $^1\text{O}_2$ acts a potential self-decontaminating additive for a variety of materials.⁵² The oxidation of chemical-warfare agent simulants to less-toxic compounds allowed for the decontamination of the surface using only oxygen from the air and visible light.

15.2.2. Photosensitizers Bound to the Nanofiber Surface

15.2.2.1. Preparation and Properties. Post-processing of electrospun nanofiber materials may allow the grafting of PS to the nanofiber surface. Singlet oxygen is thus generated externally in close proximity to a target structure that may then be efficiently photo-oxidized (Figure 15.6). The external binding of the PS also allows the modification of the surfaces of bulk materials. For example, photosensitive cotton fibers were obtained by grafting porphyrins to the fibers, and these functionalized samples exhibited antibacterial activity.⁵³ Other examples are reviewed elsewhere.⁹ In contrast to the materials with encapsulated PSs, the components of surrounding solutions may considerably quench the excited states of grafted PSs and/or induce PS photodegradation, and thus affect the ability of the PS to photogenerate $^1\text{O}_2$.

Cation-exchange nanofiber materials were prepared by electrospinning polystyrene and subsequently sulfonating the nanofibers.⁵⁴ The photoactive layer of PS was obtained by adsorbing cationic 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (TMPyP) on the nanofiber surfaces (Figure 15.6(A)). The material consists of a polymer core and a shell that can be

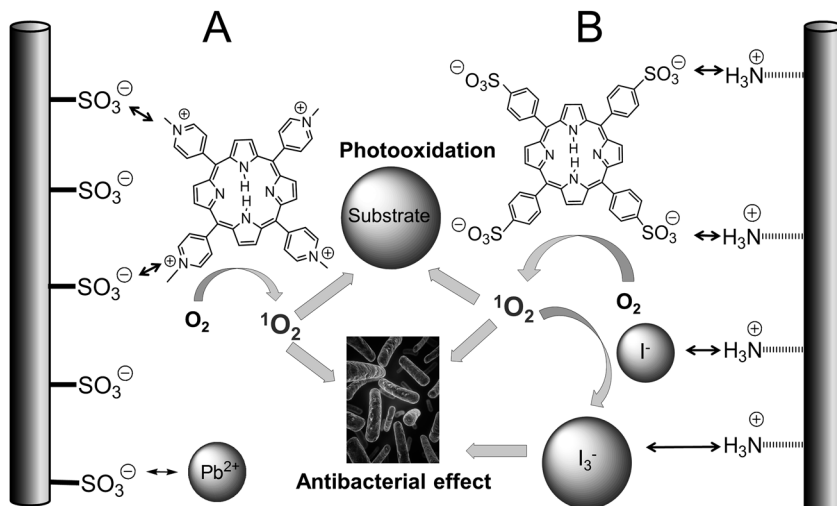


Figure 15.6. Schematic structure of cation- (A) and anion-exchange (B) nanofiber materials containing adsorbed porphyrin PSs. Photo-oxidation of a target molecule or antibacterial effect is caused by photoproducted $^1\text{O}_2$. The antibacterial effect can be even stronger on the surface of anion-exchange nanofiber material (B) with remaining free charges saturated by I^- . This efficiency was attributed to the photo-oxidation of I^- by $^1\text{O}_2$ to produce I_3^- . The presence of *in situ* photogenerated I_3^- ensured that the surfaces remained antibacterial even after the irradiation was terminated.

chemically tailored for the desired application; example applications include the rapid and selective adsorption of heavy-metal cation contaminants or organic pollutants or the killing of bacteria by photoproducted $^1\text{O}_2$. The properties of the material strongly depend on the TMPyP loading because of porphyrin aggregation on the surface. At low loadings, the decay curves of the triplet states of TMPyP were best analyzed using a double-exponential model with a considerable fraction of the TMPyP triplets accessible to oxygen. The rest of the TMPyP triplet states were not quenched by oxygen at all. The contribution of the TMPyP triplets inaccessible to oxygen increased considerably at high loading levels. Despite the fact that TMPyP-nanofiber materials generated $^1\text{O}_2$ with a rather low lifetime of approximately 1 μs , their photo-oxidation activity in aqueous media was comparable to the nanofiber materials with encapsulated TPP.³⁵ A plausible explanation for this lies in good contact between the sulfonated, hydrophilic surface and the oxidized molecules.

Anion-exchange polystyrene nanofiber materials were prepared by the two-step functionalization of the nanofiber surface by chlorosulfonic acid at ambient temperature followed by the reaction with ethylenediamine.⁵⁵ The photoactivity of these materials was introduced *via* the adsorption of 5,10,15,20-tetrakis-(4-sulfonatophenyl)porphyrin (TPPS) on the nanofiber surface (Figure 15.6(B)).

15.2.2.2. Applications. Both types of ion-exchange nanofiber materials with attached porphyrin PSs (Figure 15.6) exhibited photoantibacterial activity.^{54,55} Bacterial growth was especially inhibited on the irradiated surfaces of anion-exchange nanofibers with sorbed TPPS.⁵⁵ Typically, the average number of bacterial colonies decreased to less than 1% after 15 min of irradiation by a solar simulator. This significant antibacterial effect was attributed to the efficient photogeneration of $^1\text{O}_2$, the hydrophilicity of the nanofiber surface ($\text{ACA} \leq 5^\circ$), and the electrostatic attraction between the positively charged surface of the nanofibers and bacteria because bacterial cell surfaces possess a negative charge from the presence of phosphoryl and carboxylate substituents on outer cell envelope macromolecules.

A stronger photoantibacterial effect was observed on nanofiber surfaces with grafted TPPS and on which the remaining free surface charges were saturated by Γ^- . This efficiency was attributed to the photo-oxidation of Γ^- by $^1\text{O}_2$ on the surface of the nanofibers to produce I_3^- with strong antibacterial activity.⁵⁶ The presence of *in situ* photogenerated I_3^- (Figure 15.6(B)) ensured that the surfaces remained antibacterial even after the irradiation was terminated.⁵⁵

15.3. Polymer Nanocomposite Films

15.3.1. Photosensitizers in Layered Materials

A supramolecular PS can be prepared by the incorporation of PS molecules into the gallery of two-dimensional inorganic layered structures. Layered double hydroxides (LDHs) in particular are promising building blocks in this context due to the versatility in chemical composition of the hydroxide layers and interlayer spacing as well as to the stability and biocompatibility of this class of compounds. The general formula for LDHs is $[\text{M}_{1-x}^{2+}\text{M}_x^{3+}(\text{OH})_2]^{x+}[\text{A}_{x/n}^{n-}]^x \cdot m\text{H}_2\text{O}$, where M^{2+} and M^{3+} represent metal cations octahedrally coordinated by hydroxyl groups and A^{n-} represents an n -valent intercalated anion, which can vary from a simple inorganic species to a targeted PS anion that introduce functionality to the organic/inorganic hybrid.⁵⁷ The distance between inorganic hydroxide layers depends on the size and arrangement of the intercalated anions. PS molecules are organized in an expandable interlayer space of the LDH hosts and are separated from their surroundings. This may preserve the photophysical and photochemical properties of the introduced PS.⁵⁸ Research on the host-guest interactions of porphyrin or phthalocyanine PSs with LDHs has led to new functional nanomaterials that produce $^1\text{O}_2$ upon irradiation with visible light^{15,58} and exhibit potential in anticancer photodynamic therapy⁵⁹ and in photo-oxidation reactions.⁶⁰ These hybrid materials have also been found to be good nanofillers for the fabrication of polymer nanocomposite films with light-triggered antibacterial functionality.

15.3.2. Photosensitizers in Nanocomposite Films

The ability of porphyrins intercalated in LDHs to produce $^1\text{O}_2$ upon irradiation with visible light¹⁵ and the known cytotoxicity of $^1\text{O}_2$ led to the idea of utilizing the nanocontainer and nanofiller aspects of LDHs for the preparation of LDH-porphyrin/polymer nanocomposites for antibacterial coatings.^{61–63} In this approach, porphyrin molecules are placed in the interlamellar space of LDHs where they are partly protected from photodegradation, and nanoparticles of LDHs are dispersed in the polymer.

TPPS and Pd(II)-5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin (PdTPPC) were intercalated into LDH hosts by a coprecipitation procedure and then used as fillers in polyurethane and poly(butylene succinate), two ecofriendly polymers.⁶¹ Both X-ray diffraction and transmission electron microscopy measurements indicated that the porphyrin-LDH fillers were well dispersed in the polymer matrices and that the porphyrin molecules remained intercalated within the LDH hydroxide layers. Although LDH filler and polymers are partially phase separated, their compatibility is quite good as the polymer matrix tends to encapsulate single LDH particles and/or sheets, which is clearly observed at higher-magnification micrographs (Figure 15.7(A)). LDH/poly(butyl methacrylate) nanocomposites were prepared using LDHs, partially intercalated with TPPS.⁶² The nanocomposites produced $^1\text{O}_2$ upon irradiation and were suggested as platforms for the fabrication of antibacterial surfaces activated by visible light. Investigation of the kinetics of the quenching of the triplet states by oxygen indicated limited diffusion of oxygen within the nanocomposite (Figure 15.7(B)). Because the polymer matrix restricts the diffusion of oxygen and partially quenches $^1\text{O}_2$, the oxidative

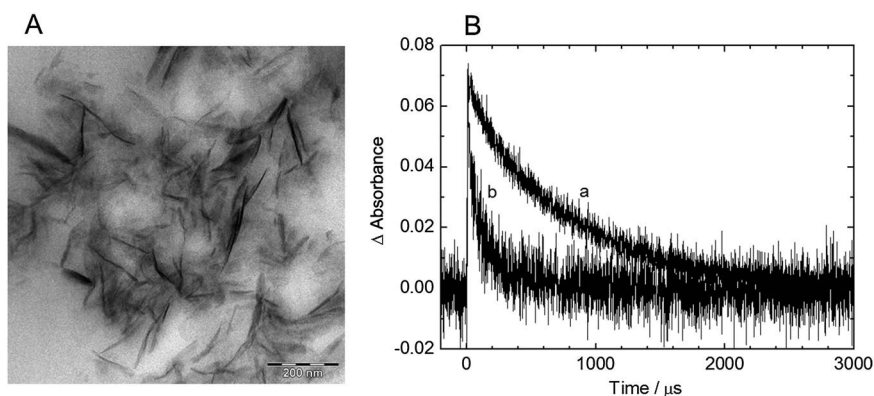


Figure 15.7. (A) Transmission electron microscopy/bright-field micrograph of the polyurethane nanocomposite with the Zn-Al LDH filler containing intercalated PdTPPC. The nanocomposite exhibits a flake structure; dark areas in the micrograph are filled with loose aggregates of LDH nanoparticles, while the light areas are composed of pure polymer. (B) Decay curves of the PdTPPC triplet states in the nanocomposite film in a vacuum (a) and in an oxygen atmosphere (b) document the quenching of the triplet states by oxygen. Adapted from ref. 61.

effect on the nanocomposite surface is due to $^1\text{O}_2$ produced within a narrow surface layer. The photostability of polyurethane composite films was studied to establish their applicability.⁶³ It was shown that the intercalation in the LDH host enhances the chemical stability of the PS by minimizing photo-bleaching and aggregation effects and that $^1\text{O}_2$ has no detrimental effects on the microstructure and viscoelastic properties of the nanocomposite.

In vitro antimicrobial tests showed that *Staphylococcus aureus* growth on the composite surfaces is inhibited by white-light irradiation. The observed total inhibition of *Pseudomonas aeruginosa* growth indicated the efficacy of the surface against biofilm formation. As noted, the advantage is that the activity of the surfaces can be tuned by adjusting the amount of porphyrin-containing filler within it. The potential applications of such photodynamic surfaces, which are sterile under white light, are of great interest in all cases in which it is desirable to maintain microbial populations at a low level.

Dye-containing polymer–mineral nanocomposites also demonstrated useful properties for construction of luminescent oxygen sensors.⁶⁴ Such systems often generate $^1\text{O}_2$; however, for this application, the effectiveness is measured by the degree of luminescence quenching by oxygen, and the formation of $^1\text{O}_2$ can have detrimental effects. The mineral phase serves as a carrier for the dye, improves the mechanical properties of the material, and may even reduce the photo-oxidation activity of $^1\text{O}_2$.

15.4. Conclusions and Prospects

Nanofiber and nanocomposite materials, when combined with PSs, can be made to produce $^1\text{O}_2$ and have antibacterial, antiviral, and photo-oxidation properties. On this basis, membranes, textiles, and coatings composed of nanofibers and nanocomposites could be excellent tools for the fabrication of functional materials that remain sterile as long as they are irradiated by visible light; such a property would be useful for the production of wound dressings, surgical masks, and self-sterile surfaces, to name a few. The most important advantages of this approach are that these materials can be biocompatible and that they are activated by sunlight and only require atmospheric oxygen as an oxidizing agent. These materials offer great versatility, and it is conceivable to design an appropriate material with additional functions for use in drug delivery or with specific performance that can be introduced by grafting or intercalating nanoparticles with cell-specific moieties or antibacterial and antiviral compounds. It should also be mentioned that, similarly to the effect on bacteria strains and viruses mentioned in this chapter, a strong cytotoxic effect can be expected towards other pathogens residing on such surfaces, including bacteria with multidrug resistance, HIV, influenza viruses, and protozoa. In addition, the porous nature of nanofiber materials allows the necessary permeation of atmospheric oxygen into the material and thus effectively protects the wound from possible infection under visible-light irradiation. The reviewed materials also have potential

use in multifunctional, chemically active filtration technologies for small-scale water-treatment systems and in the fabrication of protective clothing that is able to destroy pollutants and/or chemical-warfare agents.

The next generation of the presented materials for biomedical and environmental applications will be probably based on cost-effective and biocompatible polymers with multiple functions that can photodisinfect, decontaminate, separate and perform other functions simultaneously. Evidently, a high surface-to-weight ratio is a great benefit in the photo-oxidation of a substrate by PS-containing nanofiber materials. Still, detailed studies describing the effect of nanofiber diameter on the distribution of encapsulated PSs and the photo-oxidation efficiency are needed. It can be expected that the diameter of the nanofibers greatly influences the photo-oxidation efficiency because materials made with thinner nanofibers possess more accessible excited PS molecules and thus have higher concentration of $^1\text{O}_2$ at the surfaces.

Given the increase in the resistance of bacterial strains towards modern antibiotics, it is likely that the usage of self-sterile surfaces to control pathogens in human healthcare will be of greater importance in the near future. Developments in this field should include systematic work on the design of surfaces using a variety of (nano)materials and on understanding of the influence of structural parameters on the production of cytotoxic $^1\text{O}_2$, surface reactivity, and stability. Among the issues requiring further efforts during the coming years will be the use of biocompatible polymers and PS molecules to cover specific spectral regions for excitation. The development of pre- or postprocessing functionalization techniques to incorporate additional functions to the surfaces will introduce a generation of new materials with interesting properties.

Acknowledgements

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Chapter 16

Photochemistry in Supercritical Fluids

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16.1. Introduction

A supercritical fluid was first described historically by Baron Charles Cagniard de la Tour in 1822,¹ where he defined it as a state of matter where the liquid and vapor phases were indistinguishable. Below the critical point of the substance concerned, phase transitions from liquid to gas and back to liquid can be controlled by increases in temperature and pressure, respectively. Above the critical point, defined by critical temperature T_c and critical pressure P_c , from Gibbs' phase rule it follows that changes in temperature and pressure no longer result in a phase change and there is a continuous medium; a supercritical fluid.

Supercritical fluids are of considerable interest as solvents mainly since their physical parameters such as viscosity and density (and consequently diffusion coefficients) can be modulated by changes in temperature and pressure, resulting in changes to solvent properties without changes in chemical identity.^{2,3}

The interest in so-called Green Chemistry (or perhaps more usefully termed "environmentally unobtrusive chemistry") has grown substantially over recent years with emphasis moving towards low-energy, environmentally benign processes.⁴ Up until the last decade, photochemical methods had not been extensively explored in the context of green chemistry⁵ even though the environmental significance was recognized many years ago.⁶ More recently the use of solar radiation has rekindled this initial interest in photochemical syntheses,⁷ of which the photocatalytic use of singlet oxygen forms but one part. The definition of green chemistry as "the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances" was first formulated in the early 1990s, and subsequently in 1998 the Twelve Principles of Green Chemistry were formulated by Anastas and Werner as a framework for the design of new chemical products and processes, and indeed for the modification of existing ones.⁸ With reference to this, as pointed out by George *et al.*,⁹ "photochemistry provides opportunities for reducing reagent usage, reaction temperatures and improved selectivity". Coupled with the solvent-tuning possible in supercritical fluids, photochemical syntheses have great potential in this context.

This chapter will look at the generation, deactivation and reactions of singlet oxygen in supercritical fluids.

16.2. Singlet Oxygen Generation

Okamoto *et al.*¹⁰ first demonstrated photosensitized singlet oxygen generation in liquid and supercritical fluid carbon dioxide using 9-acetylanthracene as a sensitizer with nitrogen laser (337.1 nm) excitation. Detection was *via* phosphorescence at 1270 nm with a Hamamatsu InGaAs detector, although they reported the signal to be very weak. In independent work, Worrall *et al.*¹¹ subsequently published data obtained employing a similar technique,

with phenazine as a sensitizer with 355 nm excitation and a liquid nitrogen-cooled Ge photodiode detector (EO-980P, North Coast Scientific) to generate singlet oxygen in liquid and supercritical fluid carbon dioxide. Later¹² the same group demonstrated that the technique was equally applicable in supercritical fluid xenon. Quantum yields for singlet oxygen production in supercritical fluids have been shown to be comparable with those in conventional solvents,¹³ making this a convenient and efficient method of generating singlet oxygen.

Since singlet oxygen generation in supercritical fluids has, to date, been exclusively *via* photosensitization, the effect of the nature of the supercritical fluid on the quenching of a sensitizer by oxygen is clearly of interest. Okamoto *et al.*¹⁴ studied the fluorescence quenching of 9-cyanoanthracene by oxygen in supercritical fluid carbon dioxide, and compared it to the same process in conventional solvents. In the supercritical fluid, beyond the critical point the pressure dependence of the quenching rate can be successfully modeled using the same model as for the liquid, involving the pressure dependence of the radial distribution function. However, near the critical point there is an enhancement of the quenching constant relative to that predicted by the model that is interpreted in terms of local composition enhancement, with the ratio of the “effective” oxygen concentration relative to that of the bulk rising to 2.7 near the critical point.

16.3. Singlet Oxygen Decay in Supercritical Fluids

The first report of the study of the decay of photosensitized singlet oxygen in supercritical fluids was published by Okamoto *et al.*¹⁰ in supercritical fluid carbon dioxide, followed by a more extensive study by Worrall *et al.*¹¹ These demonstrated a remarkably long lifetime of singlet oxygen in supercritical fluid carbon dioxide of 5.1 ms at 41 °C and a pressure of 150 kg cm⁻², well above the critical point ($T_c = 32.5$ °C and $P_c = 72$ kg cm⁻²). Okamoto *et al.*¹⁰ observed that the pressure dependence of the observed singlet oxygen lifetime was much greater in the supercritical fluid than in liquid carbon dioxide over the same pressure range (at temperatures of 35 and 25 °C, respectively).

Worrall *et al.*¹¹ demonstrated that the lifetime of singlet oxygen in supercritical fluid carbon dioxide is a sensitive function of a number of factors including temperature, pressure and oxygen concentration, and that in some circumstances sensitizer concentration and singlet oxygen concentration may also play a part. Under their conditions, the observed singlet oxygen decay rate was independent of sensitizer and singlet oxygen concentration, so rate constants for these processes were not extracted. Their data demonstrated that along a given isobar, the rate constant for singlet oxygen deactivation decreased with increasing temperature, and along a given isotherm increased with increasing pressure. The rate of increase along a given isobar is more marked near the critical point as illustrated in Figure 16.1. It was noted, perhaps surprisingly, that there was no abrupt change in the

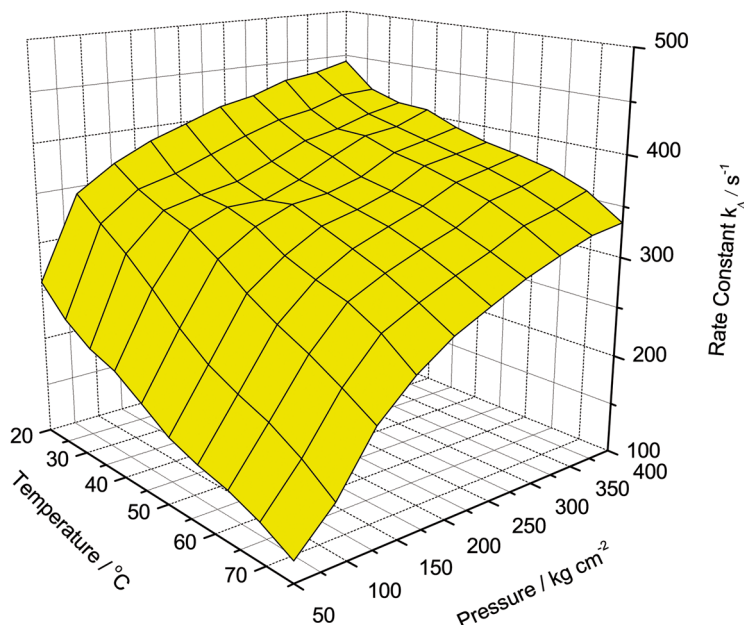


Figure 16.1. 3D plot showing the dependence of the rate constant for the decay of singlet oxygen ($\text{O}_2(\text{a}^1\Delta_g)$) in supercritical fluid carbon dioxide, k_A , on temperature and pressure. Reprinted with permission from D. R. Worrall, A. A. Abdel-Shafi and F. Wilkinson, *J. Phys. Chem. A*, 2001, **105**, 1270. Copyright (2001) American Chemical Society.

trend or values of singlet oxygen lifetimes at the transition between liquid and supercritical carbon dioxide. More recently Ogilby *et al.*¹⁵ demonstrated temperature effects on the solvent-dependent rate constant for nonradiative deactivation of singlet oxygen, and interpreted these in terms of an activated process in the electronic-to-vibrational energy-transfer process.

However, the changes observed are small relative to those observed in these studies. The pseudofirst-order equation describing the decay of singlet oxygen in supercritical fluid CO_2 can be formulated as

$$k_A = k_R + k_q^{\text{CO}_2} [\text{CO}_2] + k_q^{\text{O}_2} [\text{O}_2] + k_q^{\text{S}} [\text{S}] + k_q^{\text{Q}} [\text{Q}], \quad (16.1)$$

where the superscripts CO_2 , O_2 , S and Q indicate quenching by carbon dioxide, oxygen and sensitizer, respectively, and $[\text{S}]$ and $[\text{Q}]$ are the sensitizer and any additional quencher concentrations, respectively.¹¹ Also appearing in this equation is the radiative rate constant k_R . Solvent-dependent changes in the radiative rate constant have been demonstrated previously in the work of Ogilby *et al.*^{16,17} and Schmidt *et al.*¹⁸ However, the radiative rate constant makes only a small contribution to the overall observed decay rate constants, hence these effects have not been considered significant. Additionally, the absolute value of the initial singlet oxygen signal observed by Worrall *et al.*¹¹ varied little with changes in temperature and pressure.

An additional factor determining the rate of decay of singlet oxygen is the concentration of ground-state oxygen (quenching of singlet oxygen by ground-state oxygen is a spin-allowed process). In conventional solvents, quenching by ground-state oxygen usually plays little role due to solvent deactivation *via* electronic-to-vibrational energy transfer being so rapid.^{19–21} However, with the lifetime in supercritical fluid carbon dioxide being very long, quenching of singlet oxygen by ground-state oxygen (which is dependent on both temperature and pressure²²) must be taken account of. The rate constant for quenching of singlet oxygen by ground state oxygen at 314 K and 14.7 MPa has been determined²² as $1.62 \times 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, which is indicative of reaction control rather than diffusion control in the quenching process, where rate constants for diffusion control in supercritical fluid CO₂ in the same temperature and pressure range have been measured²³ to be of the order of $10^{11} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$. Hence, the quoted lifetimes are those extrapolated to zero oxygen concentration. The pressure dependence of the oxygen-quenching constant under such pre-equilibrium conditions can be explained on the basis of encounter complex formation, the associative and dissociative steps having different pressure dependencies.²⁴

In 2001, Worrall *et al.*¹² extended the study of singlet oxygen photophysics to supercritical fluid xenon. Here, the lack of receiving solvent modes leads to a very long singlet oxygen lifetime (extrapolated to zero oxygen concentration) of 22 ms at 325 K and 8.8 MPa (again well beyond the critical point), demonstrating that the heavy atom effect on the O₂(a¹Δ_g) to ground-state intersystem crossing more than compensates for the lack of solvent-receiving modes (xenon shows one of the highest spin-orbit coupling constants of any element at 6080 cm⁻¹²⁵). Following the work of Schmidt and Afshari²⁶ in conventional solvents, Worrall *et al.*¹² calculated the partitioning of vibrational energy between deactivated singlet oxygen and the quenching molecule, and demonstrated significant uptake of the electronic excitation energy into the higher overtone bands of the quenchers including carbon dioxide.

Factors determining the lifetime of singlet oxygen in both supercritical fluid carbon dioxide and xenon were studied in more detail in a later publication by Abdel-Shafi and Worrall.²² In this paper the contribution of quenching by ground-state oxygen to the overall deactivation rate constant for singlet oxygen in supercritical fluid xenon was comprehensively determined, and hence the “real” rate constant for quenching of singlet oxygen by xenon was extracted over a range of temperatures and pressures. The quenching rate constants by xenon are determined to be in the range 0.6 to $1.8 \times 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, in the temperature and pressure range 25 to 82 °C and 10 to 40 MPa. The rate constant for quenching by oxygen is seen to increase with increasing pressure, and decrease with increasing temperature. These values are, as seen in supercritical fluid CO₂, much less than diffusion control, indicating pre-equilibrium conditions where perturbation of the equilibrium constant for the encounter complex formation will perturb the observed reaction rate constant. Based on the scheme discussed by Schmidt *et al.*²⁷ for quenching of singlet oxygen by xenon, and treating the solvent as a hard-sphere fluid

rather than applying a continuum model, the equilibrium constant for contact complex formation can be described in terms of the radial distribution function of Yoshimura and Nakahara.²⁸ The pressure and temperature dependence of the quenching rate constant for singlet oxygen by xenon is qualitatively reproduced by the temperature and pressure dependence of the radial distribution function, but a more detailed analysis allows calculation of observed activation volumes in terms of the pressure dependence of the change in quenching rate constant with respect to the radial distribution function along a given isotherm. The reaction volumes can be related to the observed quenching rate constant by eqn (16.2):²²

$$\left(\frac{\partial \ln(k_q^{\text{Xe}} / g(\sigma_{\text{ox}}))}{\partial P} \right)_T = \frac{-\Delta V_{\text{en}} + \Delta V_{\text{en}}^{\text{HS}}}{RT} - \frac{\Delta V_{\text{isc}}}{RT} = \frac{\Delta V_c^{\#}}{RT}, \quad (16.2)$$

where $\Delta V_{\text{en}}^{\text{HS}}$ is the change in volume associated with hard-sphere contact complex formation, ΔV_{isc} is the activation volume associated with the inter-system crossing from $^1(\text{O}_2 \cdots \text{Xe})$ to $^3(\text{O}_2 \cdots \text{Xe})$ and $\Delta V_c^{\#}$ is the observed reaction volume.

The data are shown in Figure 16.2. Measured activation volumes decrease with increasing temperature and pressure, which effect is ascribed to changes in isothermal compressibility that magnify effects due to attractive and repulsive forces near the critical point. The treatment was extended to include supercritical fluid carbon dioxide, where activation volumes are larger than at the corresponding temperatures and pressures in xenon, suggestive of greater solvent–solute interactions in this fluid. In the work of Okamoto

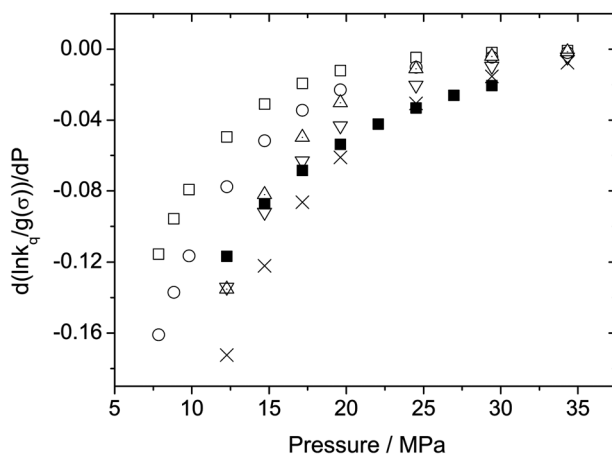


Figure 16.2. Comparison of observed activation volumes in supercritical fluid carbon dioxide and xenon, Xe 25 °C (□), Xe 35 °C (○), Xe 50 °C (Δ), Xe 60 °C (▽), Xe 82 °C (×), CO₂ 41 °C (■). Reprinted from A. A. Abdel-Shafi and D. R. Worrall, Photosensitized production of singlet oxygen and factors governing its decay in xenon and carbon dioxide supercritical fluids, *J. Photochem. Photobiol., A*, 2007, **187**, 263–269, Copyright (2007), with permission from Elsevier.

et al.,¹⁰ activation volumes were also calculated at a single temperature and pressure but without correcting for either quenching by ground-state oxygen or for the radial distribution function; however, the conclusions are in qualitative agreement. In these analyses it was assumed that, as in liquid organic solvents, the activation volume associated with intersystem crossing is approximately zero.^{27,29} These results are in agreement with the local composition enhancement observed for oxygen quenching of 9-cyanoanthracene in supercritical fluid carbon dioxide.¹⁴

Similar conclusions were drawn in a study by Okamoto *et al.*³⁰ of singlet oxygen deactivation in a different supercritical fluid, supercritical fluid ethane, using 9-acetylanthracene as a sensitizer. Larger apparent activation volumes were observed for the bimolecular quenching rate constant at lower pressures than at higher pressures in both the liquid and supercritical regions, the activation volumes being larger in the supercritical fluid. The model that was successfully applied to the liquid (derived from the encounter complex formation between singlet oxygen and the solvent and the radial distribution function) was found not to be valid in the supercritical fluid at lower pressures. This was again interpreted in terms of local density augmentation in the lower-pressure region, with the local density enhancements around the 9-acetylanthracene (based on the pressure dependence of the peak in the absorption spectrum) being very similar to those around singlet oxygen (based on the pressure-dependent decay rate).

In summary, a number of factors control the decay of singlet oxygen in supercritical fluids. These include quenching by ground-state oxygen; the nature of receiving vibrational modes in the solvent or, in the case of xenon, spin-orbit coupling effects; and the radial distribution function of hard-sphere solvent molecules around the solvent, which is perturbed by specific solvent-solute interactions around the critical point but that well describes the variation of the quenching rate constant with temperature and pressure far from criticality. From these studies a consistent picture of singlet oxygen deactivation in supercritical fluid solvents emerges even accounting for the rather different natures of the solvents themselves.

16.4. Singlet Oxygen Reactions in Supercritical Fluids

Following on from the various studies of singlet oxygen production and decay in supercritical fluids, the long lifetimes of singlet oxygen described above coupled with the lower viscosity and hence greater diffusion coefficients in supercritical fluids led to these fluids being investigated as media in which to conduct singlet oxygen-mediated oxidations, motivated in part by the implication of both singlet oxygen and superoxide in the biosynthesis of some natural products.³¹ Additionally, products can be readily recovered from the fluid simply by release of pressure.³²

The first report of homogeneous photocatalytic oxidation *via* singlet oxygen in a supercritical fluid was by George *et al.*,⁹ where they studied the

oxidation of α -terpinene to ascaridole in supercritical fluid carbon dioxide using porphyrin-based sensitizers, both tetraphenylporphyrin and a perfluorinated analog designed to improve sensitizer solubility in the supercritical fluid. Under their reaction conditions (140 bar with 1.31 mol% O₂ and irradiation with a filtered 300 W Xe arc lamp) quantitative conversion of the substrate to product was observed within 160 s as followed by FTIR and ¹H NMR. The limiting factor in determining the rate of conversion was found to be the rate of light absorption rather than mass transport in the fluid.

This initial paper was followed up by the same group³³ in 2009 where reaction scale-up was presented, necessitating a switch from a batch to a continuous-flow process in order to increase reaction volume without significant increase in pressure vessel size or optical pathlength.³⁴ Their design consisted of a sapphire tube reactor irradiated by eight 1000-lumen LEDs with an irradiated volume of 4.1 ml. Quantitative conversion of α -terpinene to ascaridole was achieved with a single reactor pass. The applicability of the approach was further demonstrated by the quantitative conversion of citronellol to its two hydroperoxide products, again with a single reactor pass. The latter reaction is interesting since the hydroperoxide products are insoluble in the supercritical carbon dioxide under the reaction conditions, 180 bar and 55 °C, but conversion is still quantitative under these biphasic conditions. In the same year, Poliakoff and George³⁵ also published further studies using tetraphenylporphyrin (TPP), 5,10,15,20-tetrakis(pentafluorophenyl)-porphyrin (TPFPP), methylene blue and rose bengal as sensitizers. As in their previous studies, TPFPP was chosen as the perfluorinated phenyl groups convey enhanced supercritical carbon dioxide solubility relative to TPP. In order to attempt to solubilize TPP, methylene blue or rose bengal they used fluorosurfactants (Krytox and Fluorolink) to enhance solubility, although this approach was not successful in dissolving high concentrations of rose bengal. A slight drop in turnover frequency was seen in the TPP/surfactant system relative to TPFPP, although as was pointed out, the TPP/surfactant system is significantly cheaper so may be more likely to find wide applicability. TPFPP was used as the photosensitizer in the oxidation of 2,3-dimethyl-2-butene *via* a Schenk “ene” (2 + 2) reaction to yield 3-hydroperoxy-2,3-dimethylbut-1-ene, and demonstrated quantitative conversion. The flexibility of this approach to peroxide formation was demonstrated using the same sensitizer to sensitize the oxidation of 1-methyl-cyclohexene, a reaction that is highly solvent dependent.³⁶ They showed that the product distribution obtained in supercritical fluid carbon dioxide is similar to that obtained in reactions performed in *n*-hexane solution. This study³⁵ also investigated the use of cosolvents to overcome the issue of poor solubilities of sensitizers or reactants; since as supercritical fluid carbon dioxide is non-polar, polar protic organic compounds typically show poor solubility. The system investigated was the singlet oxygen-mediated oxidation of 1-naphthol to naphthalene-1,4-dione, a model for the production of juglone (5-hydroxy-1,4-naphthalene-1,4-dione) from 1,5-dihydroxynaphthalene,³⁷ an important intermediate in the synthesis of biologically active natural products.³⁸ Using

dimethyl carbonate as a cosolvent and TPFPP as sensitizer they observed a much lower turnover frequency than in previous reactions, which they attributed to dilution and quenching effects by the cosolvent. The same group extended their study of flow reactions for potential scale-up to include immobilized photosensitizers.³⁹ By incorporating into PVC and aerogel supports, they demonstrated that these immobilized sensitizers could show good activity in continuous-flow reactors for both α -terpinene and citronellol oxidations, importantly without the need for a subsequent step to separate the photosensitizer from the products. Additionally, this approach negates the need for cosolvents with non-CO₂ philic photosensitizers.

A further approach to continuous photo-oxidation in supercritical fluid CO₂ is to employ biphasic catalysis to allow rapid recycling of the photocatalyst.⁴³ Here there are some quite specific requirements, in that the photocatalyst needs to be soluble in both supercritical CO₂ and also in a fluoruous solvent (which itself needs to be CO₂ soluble), so that when the pressure is released the photosensitizer preferentially dissolves in the fluoruous solvent, in which the reaction product is insoluble. Hence, the photosensitizer can be readily separated from the product and recycled around the system. Under supercritical conditions the components form a single phase in which the photoreaction takes place. This approach has been shown to have the potential to drastically reduce the amount of photocatalyst required to produce a given quantity of product. This approach has been successfully applied to the oxidation of an allylic alcohol to its corresponding hydroperoxide, a key step in the synthesis of antimalarial trioxanes.⁴⁴

It can be seen, therefore, that a number of studies have shown the potential of supercritical fluid carbon dioxide as a solvent for photocatalyzed oxidation reactions, and have demonstrated that scale-up of the process can be achieved without compromising conversion efficiencies. Challenges remain, however, particularly in overcoming solubility issues with more polar substrates and sensitizers in homogeneous photo-oxidations.

16.5. Conclusions

Overall, supercritical fluids provide a very particular medium in which to study singlet oxygen and its reactions. The very long singlet oxygen lifetimes observed in supercritical fluid carbon dioxide and xenon render it amenable to study, and the solvent tuneability allows the elucidation of effects such as local density enhancement and specific solvent-solute interactions to be observed, particularly near to the critical point. A number of chemical synthetic reactions in supercritical fluids have been reported,⁴⁰ and applications of supercritical fluids are well known in other industries such as food⁴¹ and chemical analysis through supercritical fluid chromatography.⁴² Additionally, singlet oxygen as a synthetic reagent in conventional solvents is well known. Perhaps surprisingly, therefore, there has been relatively little exploration of the applications of singlet oxygen as a synthetic reagent in

supercritical fluids. However, the desire to develop environmentally friendly processes utilizing nonhazardous materials with simple solvent recovery, coupled with the understanding which has been developed over the preceding decade or so of the behavior of singlet oxygen in supercritical fluids, may enhance interest in photocatalytic oxidations in these fluids.

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Chapter 17

Remote Singlet Oxygen Delivery Strategies

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17.1. Introduction

This chapter addresses the issue of the delivery of singlet oxygen [$^1\text{O}_2$ ($^1\Delta_g$)]. Because $^1\text{O}_2$ is highly reactive, its transport is limited to nanometer or millimeter distances depending on whether it is in solution or air. Singlet oxygen's transport is dictated by the surrounding physical and chemical quenchers, while the storage and release of $^1\text{O}_2$ by compound carriers can effectively increase its diffusion distance.

We aim to *push* the idea of $^1\text{O}_2$ delivery further (*pun intended*) by examining the following question. How do environmental conditions and molecular capture and release govern $^1\text{O}_2$ delivery to remote sites? Such a question is not just of theoretical interest and relates to the mechanistic and interfacial control of $^1\text{O}_2$ delivery whether it is to cell membranes or within molecules themselves for regioselective organic synthesis.

17.2. Background

Much focus of $^1\text{O}_2$ chemistry has been to understand how its lifetime (τ_A) and reactivity depend on the surrounding environment.^{1,2} Databases are available with $^1\text{O}_2$ decay rate constants³ and quantum yields for the formation of $^1\text{O}_2$ (Φ_A).⁴ Proof of singlet oxygen's existence is in a variety of oxidation reactions^{5,6} and in its detection by NIR,⁷⁻⁹ fluorescence,^{10,11} and EPR methods.^{12,13} Physical quenching is the “innocent” conversion of $^1\text{O}_2$ to $^3\text{O}_2$ and is distinct from chemical quenching, which is the $^1\text{O}_2$ oxidation of substrates.¹⁴⁻¹⁷

The lifetime of $^1\text{O}_2$ is diverse and spans a wide range, even in solution. Some solvents are efficient quenchers of $^1\text{O}_2$, while others increase its chances of a longer lifetime. The diffusion radius, measured to 5% $^1\text{O}_2$ remaining after three lifetimes, ranges from nanometers up to micrometers.¹⁸ A brief set of examples provides a sense for the range of lifetimes in solution: the τ_A in CCl_4 (59 ms) is longer than in toluene (30 μs), and in D_2O (65 μs) it is longer than in H_2O (3.5 μs). For H_2O , its O–H bond oscillators quench $^1\text{O}_2$ to ground-state $^3\text{O}_2$ to keep it from venturing far.¹⁹ Exchanging a protio solvent for a deutero solvent has a predictable outcome on τ_A and substrate oxidation.²⁰ The 20-fold lifetime increase of $^1\text{O}_2$ in H_2O vs. D_2O is diagnostic of its presence,²¹ as well as its chemical reactivity and toxicity increase.²²⁻²⁵

The remainder of this chapter is divided into 2 parts: first, through-space $^1\text{O}_2$ systems and interface crossings; and secondly, structure dependence for capture and release of $^1\text{O}_2$. We begin with a discussion of through-space $^1\text{O}_2$ delivery. Except for examples we feel are historically interesting, most of the literature in this chapter is cited from the past 10 years.

17.3. Through-Space $^1\text{O}_2$ Systems

One mode of $^1\text{O}_2$ delivery is through space, as a gas transported between two solids as is described next. We first describe the production of external $^1\text{O}_2$ in the gas phase in an experiment carried out 85 years ago.

17.3.1. Historically Interesting Example

Experiments showed that a volatile diffusible oxidant was transported between silica gel beads.^{26,27} Figure 17.1 shows the experiment where a sensitizer (trypaflavine) and an oxygen-trapping compound (leucomalachite green) were adsorbed separately on large and small SiO_2 gel beads, respectively. Upon mixing these beads and irradiating in the presence of O_2 , leucomalachite green was oxidized to give malachite green and eventually carbonyl compounds for clear mechanistic evidence of a diffusible $^1\text{O}_2$ species.

Currently, the notion of $^1\text{O}_2$ as a diffusible intermediate is commonplace.^{28,29} For example, recent studies^{30,31} have been carried out for generating external $^1\text{O}_2$ on a sensitizing TiO_2 surface with a terrylenediimide oxygen acceptor adsorbed on another surface. Here, a 1 mm gap between the surfaces was traversed in the gas phase.

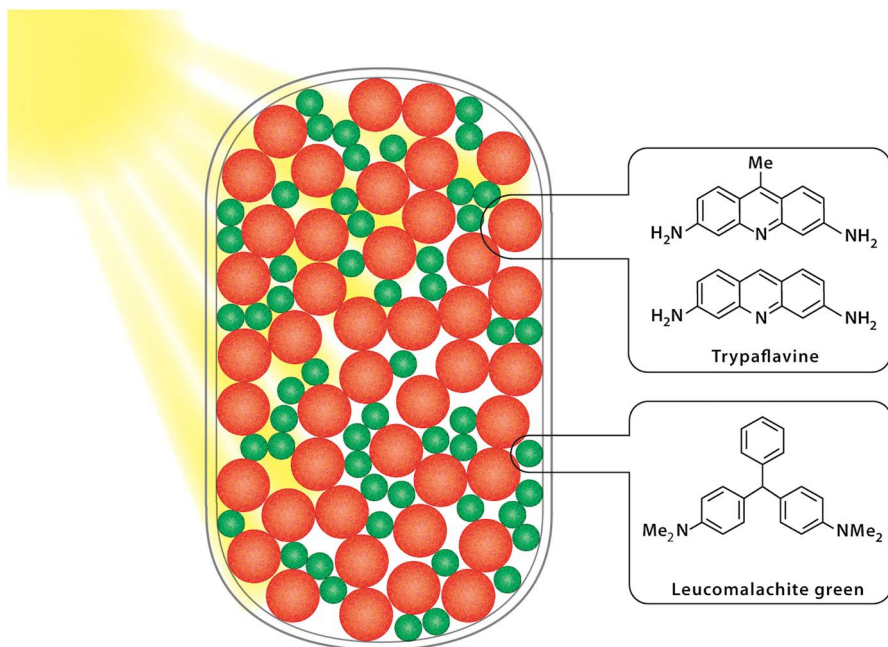


Figure 17.1. Silica gel bead experiment indicating the production of a diffusible $^1\text{O}_2$ species.

17.3.2. Clean External $^1\text{O}_2$

Methods for the generation of clean external $^1\text{O}_2$ have been developed.³² Figure 17.2 shows a method with a Pyrex tube coated with rose bengal sensitizer system that flows $^1\text{O}_2$ upon irradiation. Singlet oxygen is transported through space ~ 1.5 mm due to a 54 ms lifetime and 30 L min⁻¹ flow rate. This means that it is possible to carry $^1\text{O}_2$ out of the end of the Pyrex tube. A good historical comparison is the method of Paneth and Hofeditz³³ for their delivery of organic free radicals through a flow tube system.

Relatedly, a guided-ion-beam instrument has been developed that channels $^1\text{O}_2$ through a tube and operates at 15 Torr by a vacuum pump.³⁴ The $^1\text{O}_2$ travels through a tube to a scattering cell with a lifetime of more than 50 ms.

17.3.3. Singlet Oxygen's Journey from Air to Water

Another mode of $^1\text{O}_2$ delivery is as a gas from a solid (point of origin) to a liquid (endpoint). Figure 17.3 shows this type of $^1\text{O}_2$ delivery technique for an apparatus with a glass slide coated with sensitizer particles on its bottom face.^{35,36} With light coming down from above, it is a clean and pure source of $^1\text{O}_2$. A small air space between the sensitizer slide and solution is bridged before $^1\text{O}_2$ reaches the wells for substrate oxidation³⁵ and liposome oxidation.³⁷

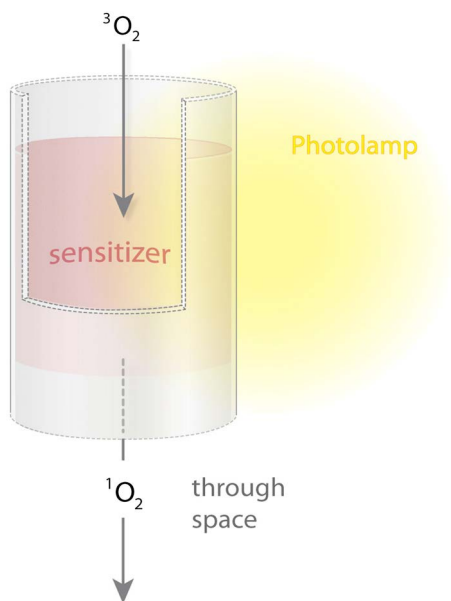


Figure 17.2. Pyrex tube coated with rose bengal system flowing $^1\text{O}_2$ upon irradiation.

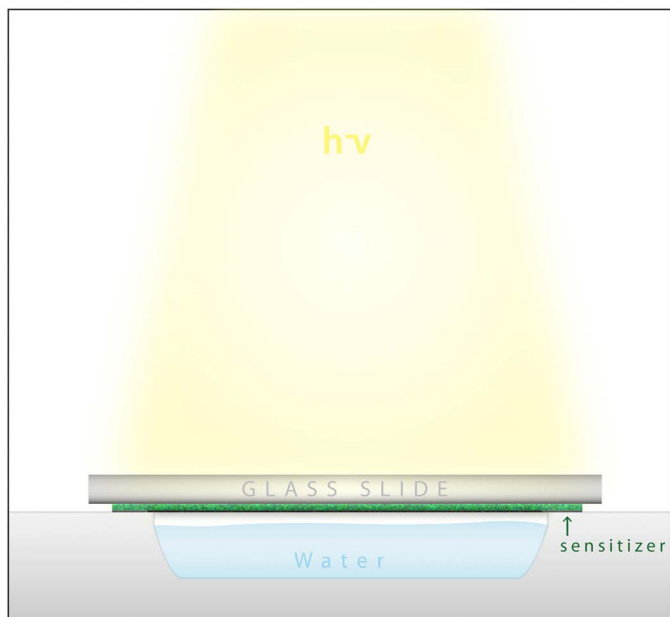


Figure 17.3. Singlet oxygen delivery from the bottom of sensitizer a glass slide through air to the top layer of water.

Making progress to understand $^1\text{O}_2$ gas/liquid interfacial chemistry, a tri-substituted olefin surfactant, 8-methylnon-7-ene-1 sulfonate, was examined in the apparatus shown in Figure 17.4.³⁸ This system emulates some of the features of the Midden system.³⁵ With red laser light coming down from above, airborne $^1\text{O}_2$ is generated below a phthalocyanine sensitizer glass slide. Airborne $^1\text{O}_2$ arrives to the surfactant at the air/water interface, and *via* an ene reaction, produces two hydroperoxides (7-hydroperoxy-8-methylnon-8-ene-1 sulfonate and (*E*)-8-hydroperoxy-8-methylnon-6-ene-1 sulfonate) in a 4:1 ratio (Scheme 17.1). This ratio points to $^1\text{O}_2$ surface activity where the alkene is oriented with methyl hydrogens pointed up in a less-solvated state than the methylene hydrogens for easier abstraction by airborne $^1\text{O}_2$. A proposed mechanism with a perepoxide transition state is shown to account for the selectivity.

17.3.4. $^1\text{O}_2$ in Bubbles, Border Crossing

Figure 17.5 shows a device in which the solution is devoid of any photosensitizers and $^1\text{O}_2$ is carried in gas bubbles.^{39,40} The lifetime of $^1\text{O}_2$ in the core of the bubble is ~ 1 ms (the bubble carried $^1\text{O}_2$ through solution a distance of 0.39 mm), but is far less ($3\ \mu\text{s}$) when fully solvated in H_2O . An N_2 presaturation encourages mass transfer of $^1\text{O}_2$ into solution. Moving into solution, an O_2 -saturated solution provides a barrier so that $^1\text{O}_2$ is less invasive. For an

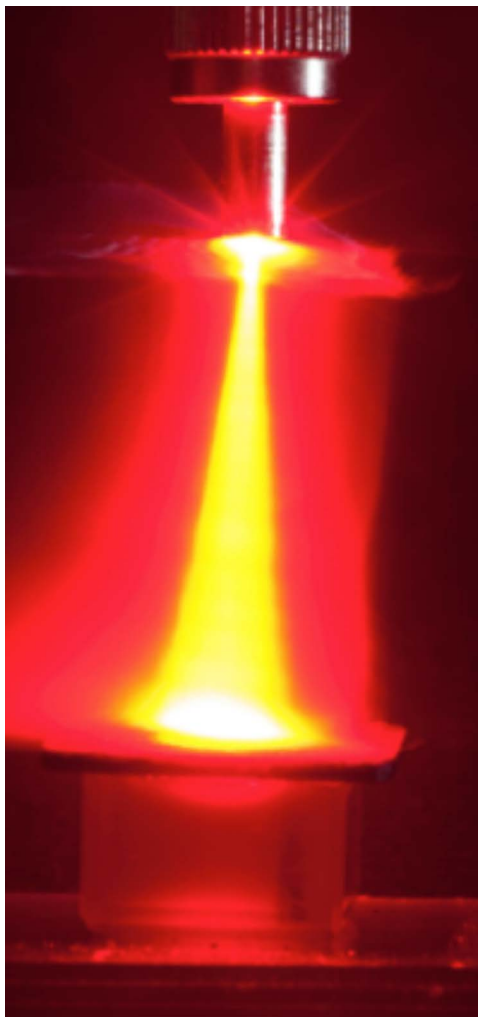
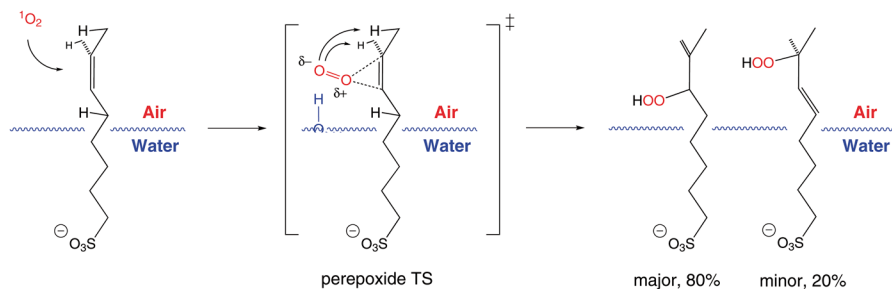


Figure 17.4. An apparatus for airborne $^1\text{O}_2$ delivery from the bottom of a sensitizer glass plate through air to an olefin surfactant at the air/water interface yielding two hydroperoxides.

O_2 -presaturated solution, instead of crossing into the water, more of the $^1\text{O}_2$ flounders at the interface in a partially wetted state.

17.3.5. $^1\text{O}_2$ Passage Through Channels

Singlet oxygen has also been delivered through the porous tip of a hollow-core fiber-optic device for localized delivery wherever the tip resides.^{41,42} The fiber optic was hollow and channeled O_2 gas and laser light to a silica



Scheme 17.1. Proposed mechanism for the reaction of airborne $^1\text{O}_2$ with an olefin surfactant at the air/water interface and preference for formation of the 7-hydroperoxy-8-methylnon-8-ene-1 sulfonate.

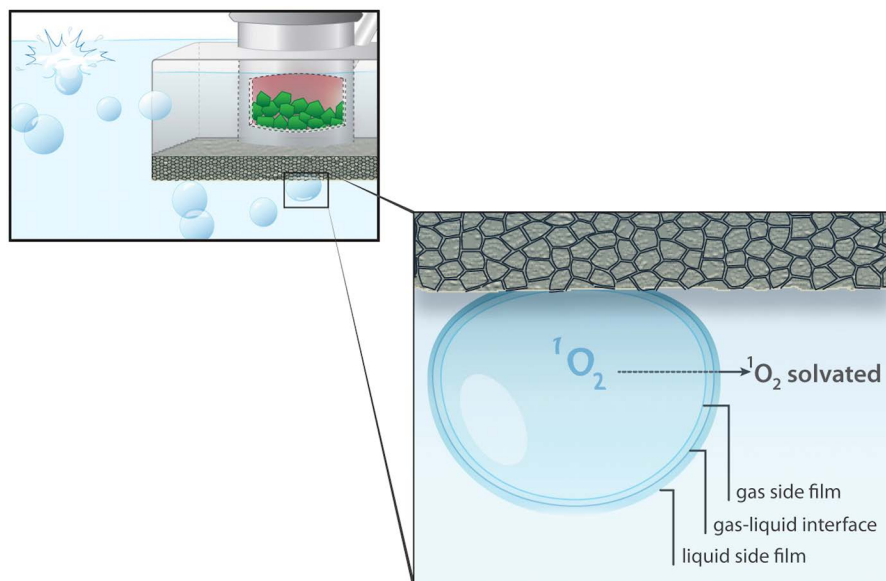


Figure 17.5. Microphotoreactor device for the delivery of gaseous $^1\text{O}_2$ to bulk solution *via* bubbles. Singlet oxygen can migrate from the core of the bubble to bulk aqueous solution constituting a “border crossing”.

tip bearing a sensitizer. O_2 transport was by a pressure gradient, where $^1\text{O}_2$ effuses through the tip. Understanding how $^1\text{O}_2$ diffuses through channels and out of pores provides key information on where oxidation events take place. Due to silanol groups the τ_{Δ} within silica pores can be lower than in neat solvent.⁴³ Relatedly, $^1\text{O}_2$ generated within a zeolite⁴⁴ was found to wriggle through and escape into bulk media.⁴⁵ To increase the amount of $^1\text{O}_2$ generated that travels through a solid, an approach can be envisaged where protective channels are provided to prevent quenching.⁴⁶ Fluorinated media help prevent $^1\text{O}_2$ quenching.^{47,48} A stabilization example is a fluorochemically

modified zeolite enabling $^1\text{O}_2$ to live longer and diffuse further.⁴⁹ Even $^1\text{O}_2$ generated within a lipid bilayer can travel and escape into bulk aqueous solution,⁵⁰ although not all of the $^1\text{O}_2$ reaches the bulk due to chemical reactions with the unsaturated fatty acids.^{51,52}

17.3.6. Quenching

Surface coating can also affect the lifetime of $^1\text{O}_2$. Similar to the zeolite system we just mentioned,⁴⁹ enhanced $^1\text{O}_2$ lifetimes have also been found within fluorinated, which silica decreased $^1\text{O}_2$ quenching compared to native silica.⁵³ Rather than conservation, additives can enhance physical quenching of $^1\text{O}_2$ by energy transfer (e.g. β -carotene) or by charge-transfer (e.g. sodium azide, hydrazines, and phenols).^{54–57} The diffusion distance of $^1\text{O}_2$ is usually reduced in the presence of such additives, although in cells, β -carotene loses its physical quenching potency.⁵⁸ The influence of an diamine-coated silica surface on decreased $^1\text{O}_2$ lifetime is also significant.⁵⁹ Sensitizer polystyrene and poly(phenylsilesquioxane) with 1,4-diazabicyclo[2.2.2]octane (DABCO) associated covalently or noncovalently have a lower total photon emissions and oxygen consumption rates indicative of improved the photostability.⁶⁰ Thus, the presence of DABCO in the polymer provided a protective action, inhibiting a chemical reaction with $^1\text{O}_2$. Such protection can increase the polymer's utility.

17.3.7. Synthetic and Biological Utility

The study of organic sensitizer polymers to oxidize compounds for synthesis has attracted much interest.^{61–70} Often polymers are postfunctionalized with sensitizer, although sensitizers can be blended in during its formation, such as in sol-gel formation. Heterogeneous sensitizers have an appeal since they are easy to separate from solution after the reaction. That is, the sensitizer can be filtered off after use. A significant body of $^1\text{O}_2$ literature is available for solution and gas phases, but less of it focuses on interfacial $^1\text{O}_2$ interactions. Killing bacteria at air/liquid, air/solid and liquid/solid interfaces is also important and $^1\text{O}_2$ offers some promise in this regard. Since developing resistance to $^1\text{O}_2$ is much more difficult for microbes to do compared to drugs, direct delivery of $^1\text{O}_2$ could offer advantages in understanding of bacteria such as multiresistant microbes. With an aim of biofilm photoinactivating polymers, cationic oligo(arylene-ethynylene)s polymers have been synthesized and tested.⁷¹ With UVA radiation in the presence of O_2 , the polymer was found to eradicate *E. coli* biofilms at levels comparable to that found for standard antibiotics such as kanamycin.⁷¹ *E. coli* exposed to these polymers showed damage indicative of $^1\text{O}_2$ delivery to cell walls.⁷²

Let us shift our focus from $^1\text{O}_2$ transport at interfaces and in homogeneous and heterogeneous media to the storage, potential transport and release of $^1\text{O}_2$ in compounds.

17.4. Reversible Capture and Release of $^1\text{O}_2$

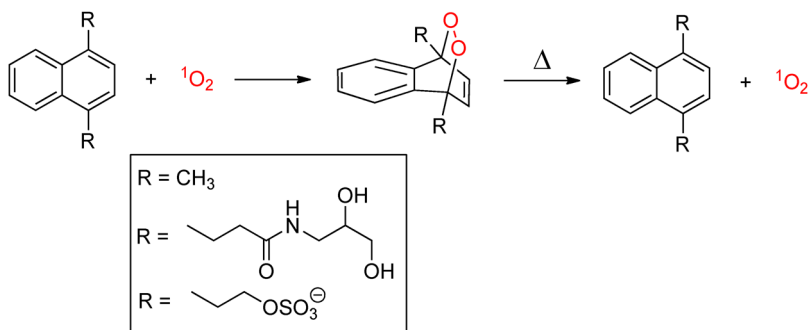
17.4.1. Idea of Temporary Storage of $^1\text{O}_2$

There is interest in understanding how $^1\text{O}_2$ can be stored in compounds to eventually release it back. It could be debated whether the capture and release of $^1\text{O}_2$ is useful for long-range transport since these compounds are noninnocent peroxides and can lead to unwanted side reactions. Furthermore, deoxygenated byproducts would remain at the site of interest upon $^1\text{O}_2$ release. However, some would agree that compound capture and release offer unique $^1\text{O}_2$ delivery opportunities. Carrier compounds exist, studied examples are substituted naphthalenes and anthracenes which can add and eliminate $^1\text{O}_2$ (Schemes 17.2 and 17.3).^{73,74,90} We begin with arene endoperoxides.

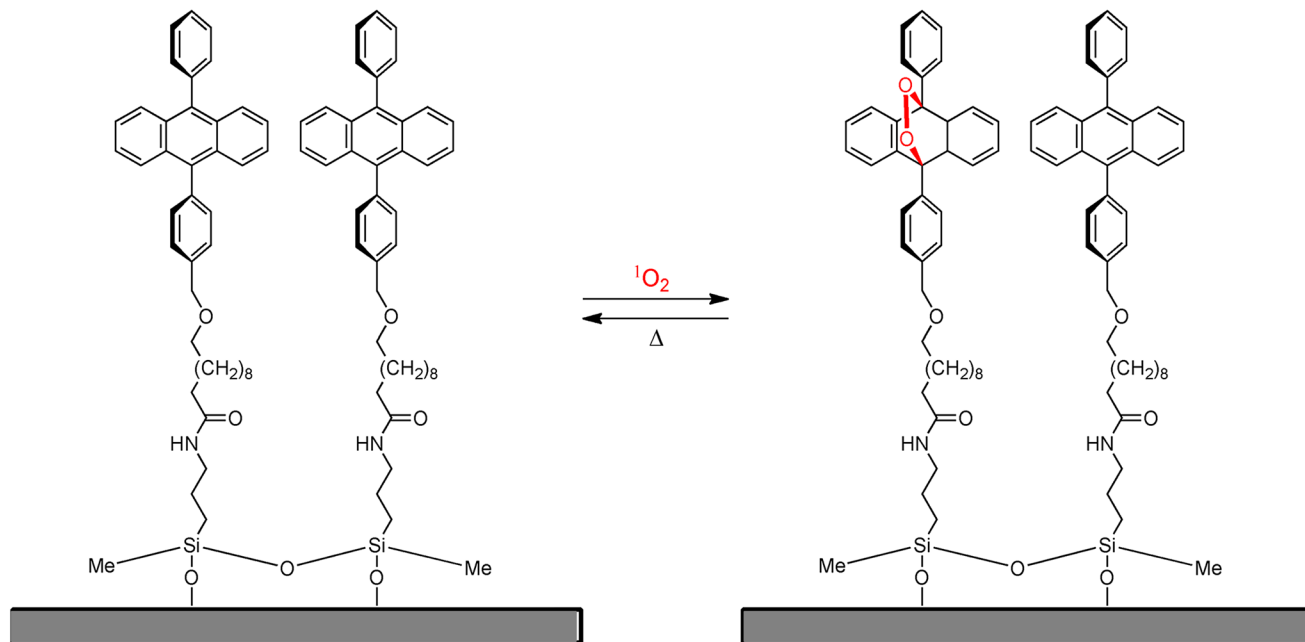
17.4.2. Arenes

The binding and release of $^1\text{O}_2$ depend on the sterics and electronics of the arene.^{73–76} Endoperoxides are 6-membered ring peroxides,^{77–80} but not all are return sources of $^1\text{O}_2$. Scheme 17.2 shows examples of 1,4-disubstituted naphthalenes found to reversibly bind $^1\text{O}_2$.^{81,82} Note that the R groups, as would be expected, tune the solubility.^{83–85} For example, the sulfonate anion side chain shown gives a water-soluble $^1\text{O}_2$ capture and release system. The capture reaction stops after the uptake of one $^1\text{O}_2$ molecule per arene, and the release of oxygen is a clean source of $^1\text{O}_2$.

Many anthracene compounds are known in the literature and their corresponding endoperoxides can release $^1\text{O}_2$ and $^3\text{O}_2$ competitively^{86–88} or undergo other reactions such as rearrangements to diepoxides.⁸⁹ Anthracenes are sensitizers for $^1\text{O}_2$ themselves, but their corresponding endoperoxides are not. Scheme 17.3 shows an example where silica-bound anthracenes reversibly bind $^1\text{O}_2$.⁹⁰ The surface anthracenes provide a high number of near-neighbor $^1\text{O}_2$ binding sites. Anthracene endoperoxides are generally more stable than naphthalene endoperoxides, and thus typically require heating to release $^1\text{O}_2$.



Scheme 17.2. Examples of substituted naphthalenes that reversibly bind $^1\text{O}_2$.

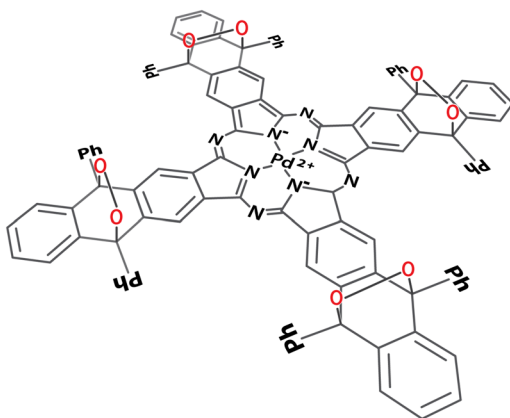


Scheme 17.3. A silica-bound anthracene that reversibly binds $^1\text{O}_2$.

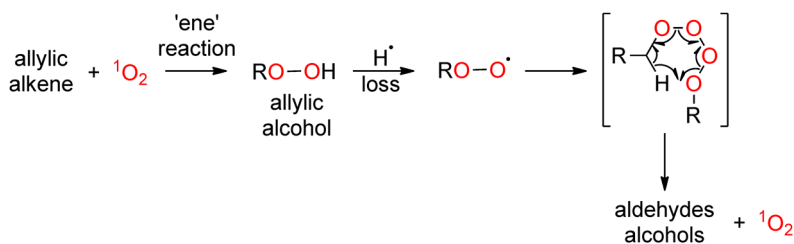
The octaphenyltetra-anthraporphyrinato palladium complex in Scheme 17.4 is a unique system that holds 4 $^1\text{O}_2$ molecules derived from a self-sensitized reaction⁹¹ All oxygen molecules can be released by two-photon absorption of 662 nm light. While the figure shows an all *cis* peroxide configuration, many configurations exist.

17.4.3. Alkenes

Alkenes containing allylic hydrogens react with $^1\text{O}_2$ to give allylic hydroperoxides *via* the ene reaction.^{92,93} Scheme 17.5 shows the resulting hydroperoxide can in turn generate $^1\text{O}_2$, as a result of peroxy radical dimerization by the Russell mechanism.⁹⁴ This is not a $^1\text{O}_2$ “carrier” reaction *per se* since $^1\text{O}_2$ adds to the alkene, which only through a series of steps regenerates $^1\text{O}_2$. The released $^1\text{O}_2$ is from a product downstream rather than the original $^1\text{O}_2$ molecule captured. While the presence of metal ions, peroxyxynitrite, HOCl and cytochrome c can facilitate the hydroperoxides to decompose and generate $^1\text{O}_2$,⁹⁵ the reaction is complex and is similar to protein photo-oxidation^{96,97} from the perspective that oxygen radicals and byproducts form in subsequent



Scheme 17.4. A unique tetra-anthracene endoperoxide complex.



Scheme 17.5. Ene reaction of $^1\text{O}_2$ with an alkene resulting in a hydroperoxide product that can regenerate $^1\text{O}_2$ after several steps.

reactions. Another example is the alkene— $^1\text{O}_2$ [2 + 2] reaction to generate dioxetanes, where their decomposition to excited-state carbonyls produce $^1\text{O}_2$ in low yields by oxygen sensitization.^{98–101}

In summary, the examples in Section 17.4 represent cases where unsaturated compounds can capture and release $^1\text{O}_2$. An aromatic compound can store $^1\text{O}_2$ as a masked species, as the arene endoperoxide. Photogenerated hydroperoxides can also function as $^1\text{O}_2$ carriers, although $^1\text{O}_2$ is returned after a rearrangement reaction. Some of these endoperoxides and hydroperoxides can be generated on preparative scales. In both cases some of the $^1\text{O}_2$ re-enters the bulk. In both cases the $^1\text{O}_2$ released can in principle diffuse and be trapped by another compound that can again release $^1\text{O}_2$ into the surrounding solution in a relay process.

17.5. Conclusion: Prospects for $^1\text{O}_2$ Delivery

Singlet oxygen is now well accepted in photo-oxidation processes and proliferation of the subject has been great. The delivery of $^1\text{O}_2$ to a remote site is a complex topic due to the dependence on environmental conditions and compounds that can carry it. Many research challenges remain including the delivery of $^1\text{O}_2$ to help guide it to sites within cells, or regioselective introduction into molecules in organic synthesis. Through mechanistic studies, new ways of delivering $^1\text{O}_2$ will emerge as our field advances.

Acknowledgements

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Section III

Reactivity of Singlet Oxygen

Chapter 18

Overview of the Chemical Reactions of Singlet Oxygen

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18.1. Practical Aspects of Running Singlet Oxygen Reactions

The ground state of dioxygen is a triplet with two parallel spins. Its reaction with closed-shell singlet organic molecules is spin forbidden. However, the lowest excited singlet state with spectroscopic designation, $^1\Delta_g$, (hereafter referred to as singlet oxygen unless indicated otherwise) reacts readily with unsaturated organic molecules in a spin-allowed process to form peroxides.¹⁻³ The success and/or rate of these chemical reactions often rely on competitive singlet oxygen deactivation channels. These deactivation channels can include nonradiative electronic-to-vibrational energy transfer to solvent or physical quenching by an added quencher, an impurity, or by the substrate itself.⁴ Singlet oxygen can also undergo a radiative decay in solution but its rate is too slow to compete with these other deactivation channels.⁵ Consequently, experimental design is critical for success.

18.1.1. Choice of Solvent

Singlet oxygen reactions are primarily conducted in solution. The choice of solvent plays a very important role in controlling the reaction and can in many cases influence both the rate and product composition. Singlet oxygen reactions are bimolecular processes as shown in Figure 18.1 and the solvent can dictate the accessible concentrations of both the substrate and singlet oxygen and insure their unfettered diffusive encounter. The efficiency of the reaction to produce the peroxide product is given by: $\text{efficiency} = (k_{\text{rxn}}[\text{Substrate}]) / (k_{\text{rxn}}[\text{Substrate}] + k_q[\text{Q}] + (k_r + k_{\text{nr}})[\text{Solvent}])$ where Q is a quencher and k_r and k_{nr} are the rate constants for radiative and nonradiative decay of singlet oxygen, respectively. To increase the efficiency of the reaction $k_q[\text{Q}] + (k_r + k_{\text{nr}})[\text{Solvent}]$ should be made as small as possible.

Efforts to increase the efficiency by reducing the magnitude of the radiative rate constant, k_r , is ineffective since it is much smaller than the nonradiative rate constant, k_{nr} , for the most frequently encountered organic solvents. A very effective way to reduce the magnitude of the more important nonradiative rate constant, k_{nr} , is to replace the hydrogens in the solvent by deuteriums. This works because the electronic energy residing in singlet oxygen is

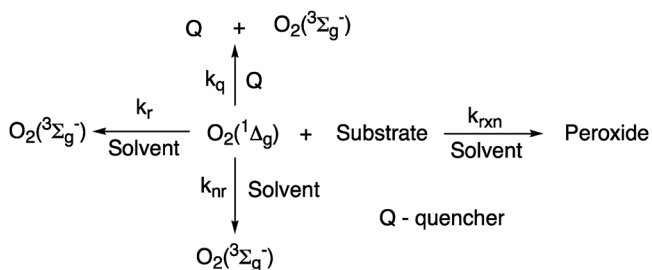


Figure 18.1. Generalized singlet oxygen reaction scheme.

Table 18.1. Singlet oxygen lifetimes.^a

Solvents	Lifetimes (μ s)	Solvents	Lifetimes (μ s)
H ₂ O/D ₂ O	3.8/62	(CH ₃) ₂ CO/(CD ₃) ₂ CO	50/723
CHCl ₃ /CDCl ₃	264/740	CH ₃ CN/CD ₃ CN	62/554
C ₆ H ₆ /C ₆ D ₆ /C ₆ F ₆	30/630/3900	C ₆ H ₅ CH ₃ /C ₆ D ₅ CD ₃	27/320

^aAverage values taken from A. A. Gorman and M. A. J. Rodgers, in *CRC Handbook of Organic Photochemistry*, ed. J. C. Scaiano, CRC Press, Boca Raton, FL, 1989, vol. II, pp. 229–247.

transferred more effectively to high-frequency solvent vibrations (e.g. O–H at 3600 cm⁻¹ or C–H at approx. 3000 cm⁻¹) than to lower-frequency solvent vibrations (e.g. C–D at approximately 2200 cm⁻¹).^{6,7} This is reflected in the dramatic increase in the lifetime ($\tau(^1\text{O}_2) = 1/k_{\text{nr}}$) of O₂(¹ Δ_g) when a protonated solvent is replaced with its C–D isotopomer^{8–10} (Table 18.1). The remarkable increase in singlet oxygen lifetime is enhanced to an even greater extent if hydrogen is replaced by fluorine (Table 18.1). In photosensitized photo-oxygenations (*vide infra*) this isotopomer/atom switch would result in a higher steady state concentration of singlet oxygen and a more rapid rate of peroxide formation (Figure 18.1). This has been referred to in the literature as the “solvent deuterium isotope trick”.¹¹

18.1.2. Choice of Photochemical or Chemical Sources of Singlet Oxygen

Singlet oxygen for synthetic applications has most often been produced by photochemical and chemical methods. These routes have their advantages and disadvantages. Photochemical routes are the most common and convenient but can be made more complex by competing hydrogen atom- and electron-transfer processes. Chemical methods are especially useful in those cases in which a well-defined quantity of singlet oxygen is needed. On the other hand, chemical routes to singlet oxygen require stoichiometric, or often more than stoichiometric quantities, of precursors whose byproducts need to be separated from the reaction mixtures, which can prove to be an expensive and laborious process.

18.1.2.1. Photochemical Sources.

Photosensitized singlet oxygenations proceed *via* quenching of low energy sensitizer triplet and/or singlet excited states.¹² The triplet-sensitized process is generally the most efficient and most often observed. The energy of the sensitizer excited state relative to the energies of the two low-lying singlet oxygen excited states (¹ Δ_g ; 22.5 kcal mol⁻¹ and ¹ Σ_g^+ ; 37.5 kcal mol⁻¹) is reflected in their population generated during the sensitization event. However, the ¹ Σ_g^+ excited state of oxygen is exceedingly short lived and does not undergo bimolecular reactions in solution and instead predominantly decays to the ¹ Δ_g state. The excellent review of Schweitzer and Schmidt⁴ should be consulted for a detailed discussion of sensitizer selection. However, some fundamental criteria for choice of the optimal singlet oxygen sensitizer include: (1) a triplet energy greater

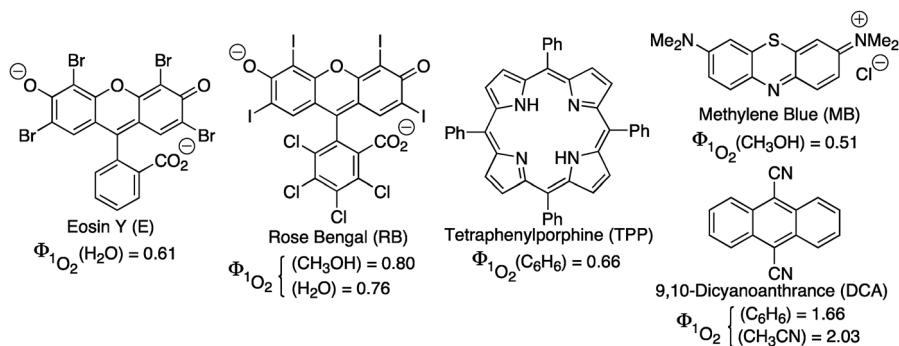


Figure 18.2. Singlet oxygen sensitizers.

than 37.5 kcal mol⁻¹; (2) lack of reactivity with singlet oxygen; (3) absence of sensitizer charge transfer complex or aggregate formation in the solvent used for the photo-oxygenation; and (4) achievable concentrations of approximately 2×10^{-4} M in the reaction medium.

Five of the most commonly used sensitizers are shown in Figure 18.2 along with their quantum yield of singlet oxygen formation in select solvents. The charged sensitizers E, RB, and MB are soluble in polar protic and polar aprotic solvents such as acetone and acetonitrile. TPP is especially useful for less-polar solvents such as benzene, chloroform, methylene chloride, carbon tetrachloride, and tetrahydrofuran. Oxygen quenching of ¹DCA* produces both ¹O₂ and ³DCA that goes on to produce a second molecule of singlet oxygen, however, it is a very electron-poor sensitizer and electron transfer processes can compete with the desired singlet oxygen reaction.¹³

Ideally, the sensitizer concentration should remain constant during the irradiation time. However, sensitizer loss by bleaching does occur in the presence of many substrates so its concentration should be high enough to capture as much light as possible without suffering detrimental inner filter effects; typically at most 2×10^{-4} to 2×10^{-3} M. Bleaching and problems associated with sensitizer removal from the reaction mixture at the end of the irradiation period, can be minimized to a certain extent by using heterogeneous sensitizers¹⁴ such as zeolite-embedded¹⁵ and polymer-bound sensitizers.¹⁶ The zeolite-embedded sensitizers are especially interesting since the singlet oxygen is primarily produced in the interior of the particle and can either react with a coabsorbed substrate or diffuse out into solution¹⁷ and react with different stereoselectivity.^{15,18} The intrazeolite *versus* bulk solution location of the substrate can be modified by proper choice of the slurry solvent.¹⁹ The intrazeolite singlet oxygen lifetimes^{17,20,21} can also be modified by choice of different zeolite photocatalysts.

18.1.2.2. Chemical Sources (Ref. 22). Historically, the oldest well-established chemical source of singlet oxygen is the reaction of sodium hypochlorite with hydrogen peroxide.²³ This reaction produces singlet oxygen in quantitative

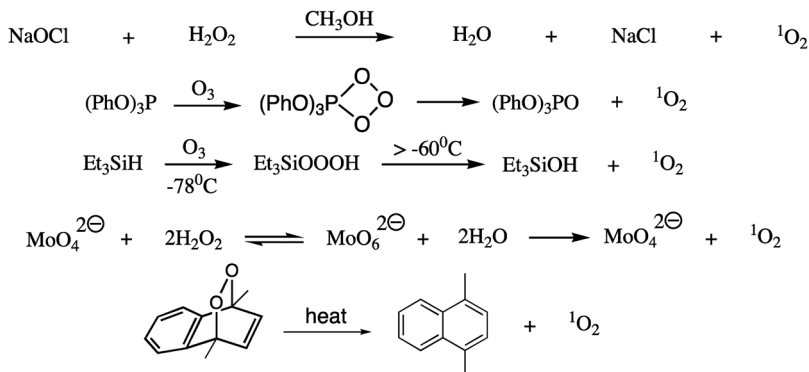


Figure 18.3. Chemical sources of singlet oxygen.

yield (Figure 18.3). The procedure, however, has limitations and cannot be used for substrates that react with hydrogen peroxide or cannot tolerate water or alcohol solvents.

Other chemical sources of singlet oxygen include decomposition of phosphite ozonides,^{24–26} hydrotrioxides,^{27–29} peroxymolybdates,^{30–32} and endoperoxides³³ (Figure 18.3). The formations and decompositions of these chemical singlet oxygen sources can be complex (Figure 18.3) and the mechanism for the delivery of dioxygen can differ as a function of reaction condition and substrate identity. For example, triphenyl phosphite ozonide³⁴ and triethylsilyl hydrotrioxide³⁵ often react directly with organic substrates at temperatures well below the temperature at which they decompose to form singlet oxygen. If the intermediacy of singlet oxygen is important for a given application that uses these chemical precursors, its formation should be unequivocally demonstrated for each reaction system.

18.1.3. Choice of Experimental Apparatus

The choice of photochemical reactor design to generate singlet oxygen is critical in order to maximize the efficiency of the reaction and to minimize unwanted byproducts. The best choice depends on the scale of the reaction. For medium to large-scale preparative reactions usually immersion or continuous-flow photochemical reactors are the best choice. The high photon collection efficiency of immersion wells is achieved by surrounding the irradiation source with the reaction mixture.³⁶ Immersion technology should be limited to medium-size preparative reactions because scale-up is difficult and safe oxygen delivery, and lamp cooling and filtering are difficult to address and can become real safety issues with increasing reactor size. On the other hand, continuous-flow reactors³⁷ either using macroscopic tubing^{38,39} or microcapillary channel embedded films are more amenable to scale-up.

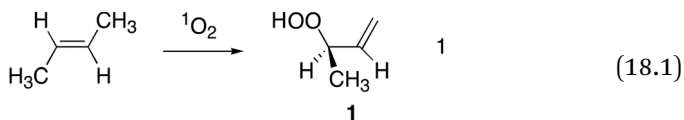
A wide variety of irradiation sources have been used to generate singlet oxygen for preparative and mechanistic purposes including medium- and high-pressure mercury, tungsten-halogen, medium-pressure sodium, high-intensity xenon, and compact fluorescent lamps, and light-emitting diodes (LEDs). Medium-pressure sodium lamps are attractive because of their high efficiency for visible-light generation. Compact fluorescent lamps⁴⁰ and LEDs⁴¹ are also attractive because they emit visible light at wavelengths that overlap with the absorption bands of the sensitizers shown in Figure 18.2. On the other hand, medium- and high-pressure mercury, tungsten-halogen, and xenon lamps all generate substantial quantities of UV photons that need to be removed to prevent unwanted absorption by substrates. This can be easily accomplished by the use of either readily available glass⁴² or solution filters, albeit with significant loss of photon flux.⁴³

18.2. Fundamental Reactions

Fundamental often encountered reactions of singlet oxygen include: (1) ene reactions; (2) 4 + 2 and 2 + 2 cycloadditions; and (3) reactions at heteroatom centers. This reactivity pattern is indicative of the electrophilic character of singlet oxygen, which consequently reacts preferentially with electron-rich substrates containing energetically high-lying, loosely held nonbonding or π -electrons on a carbon framework or at a heteroatom center.

18.2.1. Ene Reactions

The singlet oxygen ene reaction (eqn (18.1)) was first reported in the 1940s by Schenck.⁴⁴ The product of the reaction is an allylic hydroperoxide, **1**, formed by addition of oxygen to an sp^2 -hybridized carbon of the alkene substrate and abstraction of hydrogen from a distal allylic carbon. These allylic hydroperoxides can be readily reduced to synthetically important allylic alcohols. The synthetic utility of the singlet oxygen ene reaction lies in its exquisite and controllable stereo- and regiochemistry. This control is provided by: (1) mechanistic constraints; (2) electronic perturbations; (3) steric perturbations; and (4) hydrogen bonding. We will briefly introduce each of these controls below but invite the reader to consult several excellent reviews for more details.^{45–48}



18.2.1.1. Mechanistic Constraints. The addition of singlet oxygen and the abstraction of the allylic hydrogen occur from the same face of the alkene as illustrated by the formation of allylic hydroperoxides **A** and **B** rather than **C** and **D** during the ene reaction of chiral alkene, **2**⁴⁹ (Figure 18.4).

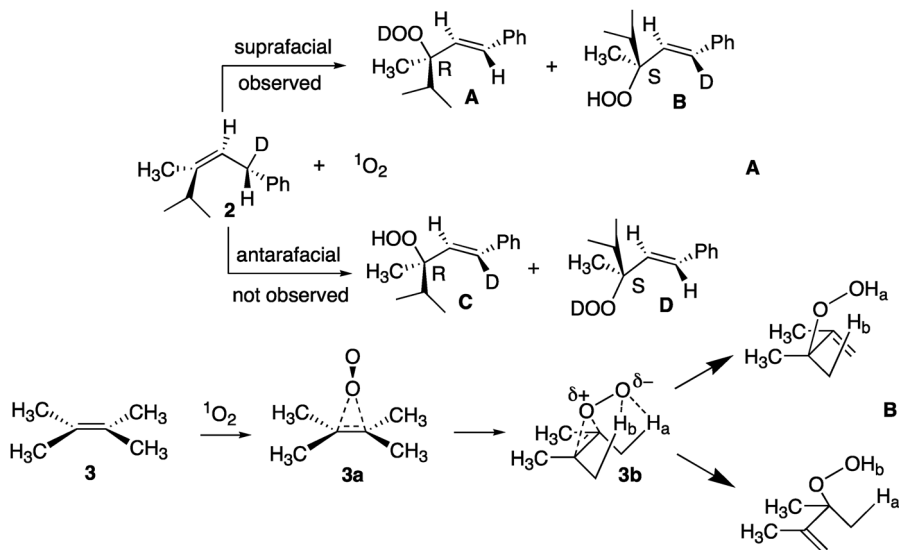


Figure 18.4. Stereochemistry, **A**, and mechanism, **B**, of the singlet oxygen ene reaction.

This stereochemistry is dictated by the mechanism and in particular by the symmetry of perepoxide **3b** (Figure 18.4) on the reaction surface for allylic hydroperoxide formation (Figure 18.4). The pendant oxygen (the partially negative oxygen) in the perepoxide, **3b**, can only abstract hydrogens **a** or **b** that are on the same face of the alkene as the oxygen molecule. The mechanism depicted for the singlet oxygen ene reaction of **3** in Figure 18.4 is generally accepted, however, the precise classification of points **3a** and **3b** on the energy surface is still a matter of debate. Elegant kinetic isotope effect studies led to the suggestion that **3a** is a transition state on the way to the perepoxide intermediate, **3b**.⁴⁹ On the other hand, high-level computational studies have suggested that the reaction is concerted and that both **3a** and **3b** are transition states on the way to the allylic hydroperoxide products.⁵⁰ Sequential formation of two transition states without an intervening intermediate is possible because transition states are saddle points, not maxima, on the reaction surface.

18.2.1.2. Electronic Perturbations. There is a preference to abstract hydrogen from the more highly substituted side of trisubstituted alkenes; a phenomenon known as the “*cis* effect”. In the case of *Z*-3-methyl-2-pentene, **4**, (Figure 18.5) this leads to 90% of **A** and **B** and only 10% of allylic hydroperoxide, **C**.⁵¹ This is consistent with the greater electronic stabilization of perepoxide **4a**, in comparison to **4b**, by electronic interactions of the pendant oxygen with two, rather than one, allylic hydrogen. The electronic character of substituents on an aromatic ring was used to influence the regiochemistry of hydrogen

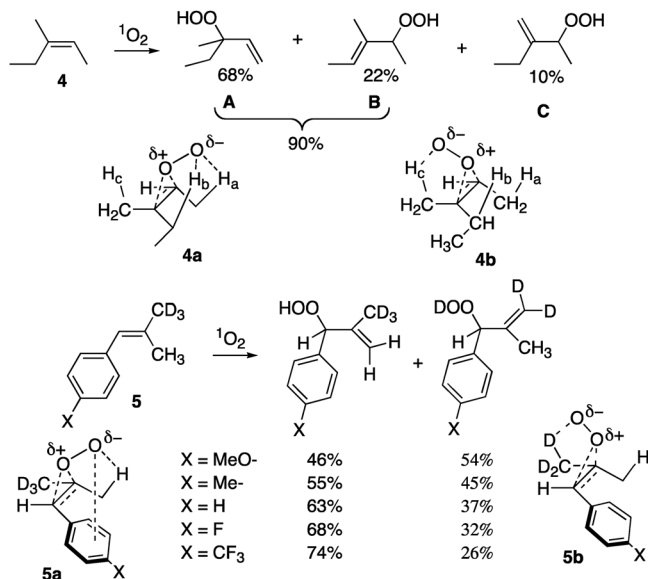
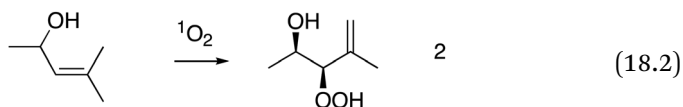


Figure 18.5. Electronic effects in singlet oxygen ene reactions.

abstraction in the singlet oxygen ene reaction of **5**⁵² (Figure 18.5). Increasing electron-withdrawing character of the substituent preferentially stabilized perepoxide **5a** relative to **5b** and directed hydrogen abstraction to the methyl group *cis* to the aryl ring.

18.2.1.3. Steric Perturbations. Steric effects can influence the geometries of both **3a** and **3b** (Figure 18.4) and as a consequence both the stereo- and regiochemistry of product formation. For example, large groups can lead to an “anti-*cis* effect” products where abstraction of hydrogen occurs predominantly from the least substituted side of the alkene.⁵³ Large substituents can also selectively lengthen one of the C–O bonds in perepoxide **3b** influencing from which end of the double bond hydrogen abstraction occurs leading to the so-called “geminal” and “large group” effects.^{45,47}

18.2.1.4. Hydrogen Bonding. Singlet oxygen ene reactions are insensitive to solvent effects. Allylic alcohols, however, are a stark exception to this generalization. Hydrogen bonding, which can be interrupted by protic solvents, can influence the diastereoselectivity of hydroperoxy alcohol formation^{54,55} (eqn (18.2)).



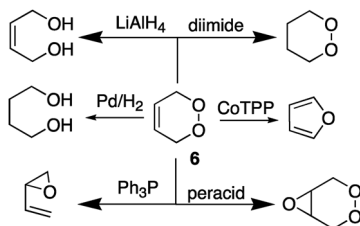


Figure 18.6. Synthetic transformations of endoperoxides.

18.2.2. 4 + 2 Cycloadditions

Electron rich *s-cis* dienes react with singlet oxygen to form endoperoxides, **6**⁵⁶ (Figure 18.6). In direct analogy to the products formed in the more familiar Diels–Alder reactions, endoperoxides are formed stereospecifically. In addition, in many cases the endoperoxides are stable and can be isolated and purified. The 1,3-diene unit can be acyclic, cyclic, polyaromatic, or heteroatomic. Aromatic endoperoxides such as 1,4-dimethylnaphthalene endoperoxide (Figure 18.3), and 9,10-diarylanthracene endoperoxides^{57,58} undergo retro-Diels–Alder reactions to release both singlet and triplet oxygen. The 4 + 2 singlet oxygenations of higher acenes such as tetracenes and pentacenes have been reported to form regioisomeric endoperoxides by both concerted and stepwise pathways.⁵⁹ The importance of the 4 + 2 cycloaddition is a direct result of the ability of the endoperoxide products to undergo a large number of useful synthetic transformations^{33,60} (Figure 18.6).

18.2.3. 2 + 2 Cycloadditions

Dioxetanes (1,2-dioxacyclobutanes) are formed during 2 + 2 cycloadditions of singlet oxygen with electron rich alkenes (Figure 18.7). Their formations in some cases can be stereospecific (*e.g.* **7a** and **7b**),⁶¹ and/or contaminated with both ene and 4 + 2 cycloaddition products.⁶² In most, but not all, cases the singlet oxygen ene reaction dominates. In those cases, however, where: (1) no allylic hydrogen is available;⁶³ (2) where the allylic hydrogens are not optimally aligned perpendicular to the π -system;⁶⁴ or (3) where an allylic heteroatom steers singlet oxygen to the side of the alkene without an available allylic hydrogen, dioxetanes can be the major products.⁶⁵

Singlet oxygen 2 + 2 cycloadditions coupled with thermal decompositions of the dioxetane products is a useful method to oxidatively cleave carbon–carbon double bonds (*e.g.* **8**) (Figure 18.7). In many cases only the cleavage products are observed and are often used to support the intermediacy of the dioxetane. This is compelling evidence when no allylic hydrogens are available for extraction⁶⁶ but not when they are present.^{67,68} In the photo-oxygenation of dihydrohexamethyl Dewar benzene, **9**, the allylic hydroperoxide is the intermediate that leads to oxidative cleavage under remarkably

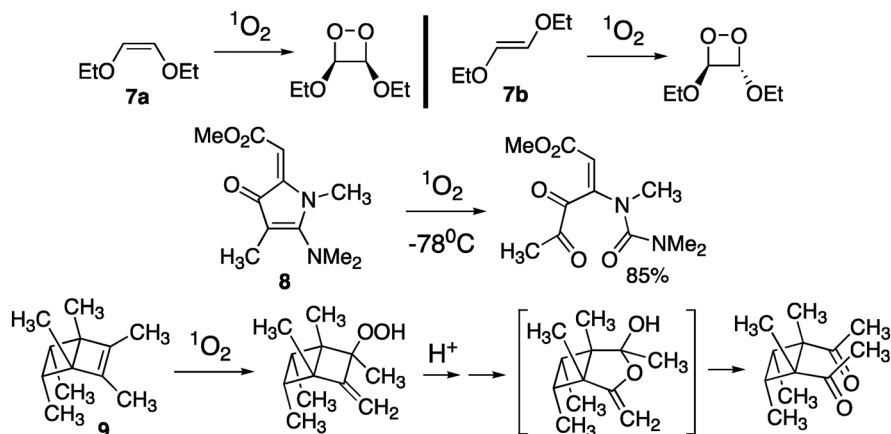


Figure 18.7. Formation and decompositions of dioxetanes.

mild conditions.⁶⁹ Careful isolation of the allylic hydroperoxide and treatment with a drop of HCl provided evidence for its viability as the key intermediate in the oxidative cleavage *via* a Hock-type reaction.⁷⁰

The mechanism of the singlet oxygen 2 + 2 cycloaddition has been explored both experimentally and computationally. Concerted,⁷⁰ and stepwise mechanisms *via* zwitterions,^{71–73} biradicals,⁷⁴ perepoxides,⁷⁵ and ion pairs,⁷⁶ have all been suggested. An electron transfer-initiated dioxetane formation that involves reaction of ground-state oxygen with an alkene radical cation has also been observed^{77–79} and should be considered as a mechanism that can potentially compete with the singlet oxygen pathway especially when electron poor sensitizers are used.

18.2.4. Reactions at Heteroatom Centers

The reactions of singlet oxygen have been studied with a variety of heteroatom containing organic molecules including: (1) organosulfur compounds;⁸⁰ (2) phosphines; (3) nitrogen compounds; and (4) a limited number of organometallic complexes. Organosulfur compounds react with singlet oxygen to give sulfoxides along with a small amount of sulfone. Formation of the sulfoxide in polar aprotic media proceeds *via* two intermediates, a persulfoxide **10a**, and a hydroperoxysulfonium ylide **10b**⁸¹ (Figure 18.8). The efficiency of the reaction is very poor (less than 5% in the case of **10**) since >95% of **10a** decompose by a physical quenching, *k'*q, route to regenerate **10** and triplet oxygen. The reaction becomes 100% efficient in alcohol solvents. The alcohol traps the persulfoxide by either hydrogen bonding or nucleophilic addition to sulfur to form a hydroperoxy sulfurane, thereby competitively inhibiting energy wasting physical quenching.⁴⁷ The reactions of singlet oxygen with trivalent phosphorus compounds have not been subjected to the same level of scrutiny as divalent sulfur compounds. Nevertheless, a careful

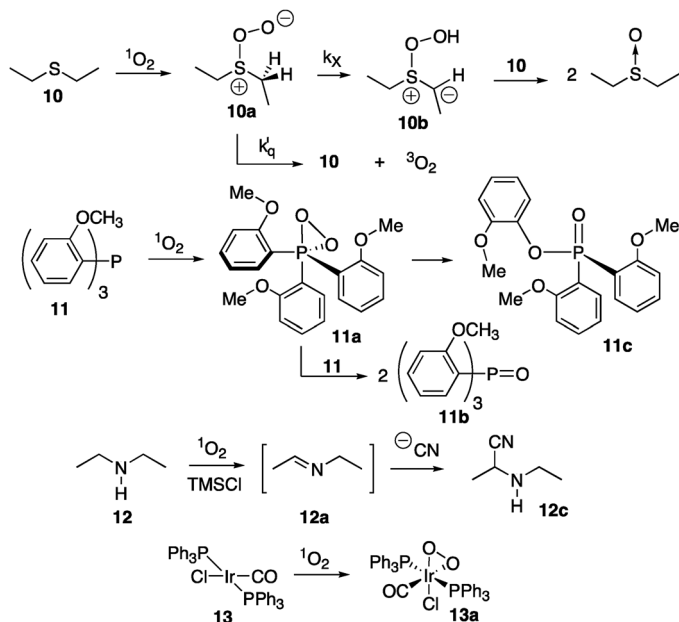


Figure 18.8. Examples of reaction of $^1\text{O}_2$ with heteroatom centers.

study of tris(*o*-phenyl)phosphine, **11**, has provided valuable insight into its reactivity as depicted in Figure 18.8.^{82–85} Only a single intermediate, the phosphadioxirane, **11b**, is kinetically required on the reaction surface, which can either be trapped by **11** or at low concentrations of **11** unimolecularly rearrange to phosphinate, **11c** (Figure 18.8). Physical quenching, by either an outer-sphere electron-transfer or charge-transfer mechanism, dominates the reactions of amines and hydrazines with singlet oxygen.^{86,87} Chemical reactions of amines with singlet oxygen have been proposed (*e.g.* **12**)⁴¹ but other possibilities have only been ruled out in a limited number of cases.⁸⁸ Finally, reactions at metal centers (*e.g.* **13**) have only recently attracted attention^{89–92} despite the fact that formation of metal-centered dioxiranes (*M*-peroxo complexes) are well established, stable, and have been observed to form under a variety of other conditions.⁹³

Acknowledgements

We thank the National Science Foundation for the support of our research.

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Chapter 19

Singlet Oxygen as a Reagent in Organic Synthesis

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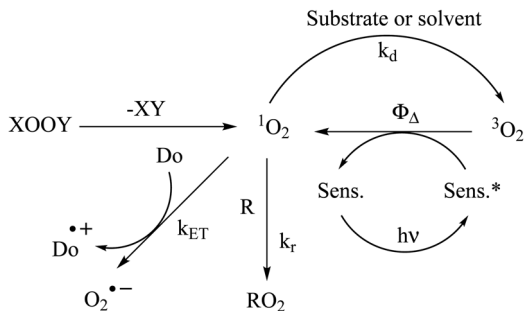
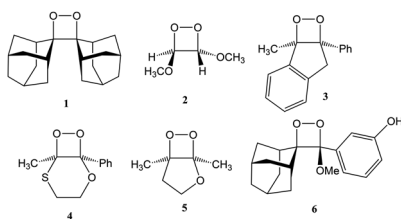
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19.1. Applications of Singlet Oxygen in Organic Synthesis

In order to apply singlet oxygen as a reliable reagent in organic synthesis,¹ several unusual properties of this molecule have to be taken into account. Singlet oxygen is our pet name for the first electronically excited state of molecular oxygen that is generated by chemical methods (*e.g.* from hydrogen peroxide,² phosphane/phosphite ozonides,³ endoperoxides,⁴ permolybdate,⁵ peracids,⁶ Caro's acid⁷) or by energy transfer from an electronically singlet or triplet dye molecule, the *singlet oxygen sensitizer*.⁸ The latter process is convenient and appears to represent an ideal green process because it uses natural dyes, oxygen from air and visible light. The lifetime of singlet oxygen depends on the reaction environment and spans from several microseconds (in water) to fractions of a second (in fluorinated solvents or in the gas phase under standard conditions).⁹ The generation of singlet oxygen is controlled by oxygen solubility, absorption properties of the sensitizer, light intensity, and the singlet oxygen quantum yield (Φ_{Δ}).¹⁰ The reactivity of singlet oxygen with organic substrates is also influenced by several factors: nonreactive collision, physical quenching and chemical quenching (k_r) are the three events that can happen in singlet oxygen–substrate collisions (Scheme 19.1). Very fast reactions can occur with rate constants k_r up to $10^9 \text{ M}^{-1} \text{ s}^{-1}$; slower reactions with rate constants around $10^5 \text{ M}^{-1} \text{ s}^{-1}$ are still synthetically feasible. Chemical reactions that are slower cannot compete with unimolecular singlet oxygen deactivation (k_d). Fast cycloaddition reactions, *e.g.* with substituted diphenylisobenzofurans or anthracenes, can be used as singlet oxygen probes that operate by a ratiometric fluorescence detection¹¹ or by fluorescence on/off switch.¹² A classical probe molecule is the terpene limonene that results in a characteristic mixture of ene products (allylic hydroperoxides) only when treated with singlet oxygen.^{13,14} In the following sections the most relevant synthetic applications of singlet oxygen reactions with organic substrates are, by no means comprehensive, illustrated by a handful of recently published examples. Typical reaction modes for chemical quenching are $2 + 2$, $4 + 2$, ene reactions, and heteroatom oxidation. Sulfur oxidation is not covered in the chapter.¹⁵ The solvent plays a crucial role for all synthetic applications of singlet oxygen, *e.g.* a strong (and useful) deuterium solvent isotope effect on k_d exists.¹⁶

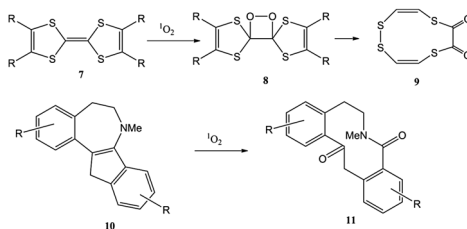
19.2. Applications of [2+2]-Cycloaddition: Dioxetane Formation

The $2 + 2$ -cycloaddition of singlet oxygen resulting in 1,2-dioxetanes is the most demanding reaction with respect to the substrate structures.^{17–19} From a thermodynamic point of view, ene reactions are much more favorable and thus, allylic hydrogens are in most cases deteriorating the dioxetane formation. The mechanism of the cycloaddition most probably involves a perepoxide intermediate in analogy to halogen addition.²⁰

**Scheme 19.1.** General reactivity pattern.**Scheme 19.2.** Typical 2 + 2 products.

Electron-rich alkenes without appropriate allylic hydrogens are the right substrates for the formation of 1,2-dioxetanes, *i.e.* enamines, enamides, enolethers, ketene aminals or ketene acetals, or even more activated alkenes such as tetrakis-enolethers or tetrakis-thioenolethers.^{21,22} A considerable number of stable dioxetanes have been prepared from alkyl- and/or aryl-substituted alkenes or from enol ethers, in many cases dioxetane formation has been postulated on the basis of the characteristic cleavage products, frequently accompanied with emission of light. Dicarbonyl compounds deriving from the cleavage of 1,2-dioxetanes are often found in the photo-oxygenation of some heterocycles as pyrroles or indoles.^{23,24} Bisadamantylidenedioxetane **1** is the most thermally stable 1,2-dioxetane (Scheme 19.2),²⁵ and also in the presence of one adamantylidene group 1,2-dioxetanes are readily formed and stabilized.²⁶ Typical representatives are shown in Scheme 19.2 involving **1**, the bis-enolether-adduct **2**,²⁷ the indene-adduct **3**,²⁸ the oxathiin-adduct **4**,²⁹ the 2,3-dihydrofuran adduct **5**,³⁰ the legendary CIEEL-chemoluminescent adamantylidene adduct **6**.³¹

Tetrathiafulvalenes **7** are highly reactive substrates for 2 + 2-cycloaddition with singlet oxygen resulting in 1,2-dioxetanes **8** that thermally cleave to ten-membered tetrathiaacyclo-1,2-diones **9**.³² Cyclic enamides were used as substrates for the synthesis of chemiluminescent 1,2-dioxetanes.³³ N-protected enamines **10** that are part of a bicyclic ring systems can be cleaved by 2 + 2-cycloaddition and subsequent 1,2-dioxetane ring cleavage. In the synthesis of protopine alkaloids this key step is applied for the formation of the central ten-membered ring **11** (Scheme 19.3).³⁴



Scheme 19.3. Thiafulvalenes and pyrroline dioxetanes.

19.3. Applications of the Ene Reaction: Allylic Hydroperoxides

For the allylic activation of C–C double bonds in the presence of allylic hydrogen atoms, the Schenck ene reaction is the most prominent path.³⁵ The reaction was first described in 1943 by Schenck.³⁶ In the course of this reaction, $^1\text{O}_2$ attacks one center of a C–C double bond with abstraction of an allylic hydrogen atom or an allylic silyl group (bound to oxygen) and simultaneous allylic shift of the double bond. As the result of this reaction, allylic hydroperoxides are formed or *O*-silylated α -hydroperoxy carbonyl compounds. Numerous reviews appeared in the last decade on the selectivity pattern of the singlet oxygen ene reaction.^{37–41} The state-of-the-art understanding of the ene reaction is a concerted process with a perepoxide-like energy plateau, *i.e.* a two-step no-intermediate reaction involving a bifurcated potential energy surface.^{42,43} Similar to other thermal ene reactions with electrophilic enophiles, this process prefers electron-rich alkenes: tetra-alkylated alkenes are favored over trialkylated substrates by a factor of 10.⁴⁴

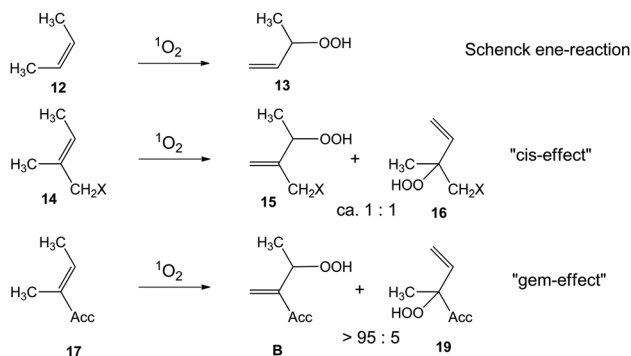
The different regioselectivity pattern, however, do not support a typical concerted process.⁴⁵ Besides the long-known “*cis*-effect” (in **14**),⁴⁶ the “*gem* effect” is most unusual because electron-poor substrates **17** (α,β -dialkylated acrylates and derivatives) add $^1\text{O}_2$ with high regioselectivity to give the allylic hydroperoxides **18**.⁴⁷ Tiglic acid derivatives result in regioisomeric hydroperoxide ratios of 95:5 up to 98:2. This effect was also observed for α,β -unsaturated nitriles⁴⁸ sulfoxides,⁴⁹ and sulfonates.⁵⁰ Also the corresponding dimethylstyrene showed *gem*-selectivity.⁵¹ The hydroperoxides **18** can be reduced to the corresponding allylic alcohols that constitute typical Baylis–Hillman–Morita products (Scheme 19.4).⁵²

The singlet oxygen ene reaction with allylic alcohols **19** delivers the corresponding β -hydroperoxy alcohols **20** with high regioselectivity and, starting from chiral allylic alcohols, also with high diastereoselectivities.^{53,54} From the primary products, by Lewis acid-catalyzed peroxyacetalization 1,2,4-trioxanes **21** are available.⁵⁵ This route was applied for the syntheses of highly antimalaria-active spirofused 1,2,4-trioxanes⁵⁶ as well as bicyclic perorthoesters.⁵⁷ The allylic alcohols have to be electron rich in order to compensate the deactivating function of the hydroxyl group on the electronically excited singlet oxygen in the initial ene reaction.⁵⁸ 4-Hydroxy tiglic acid is a moderately reactive

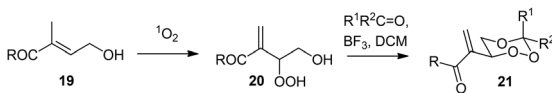
substrate, that adds singlet oxygen regioselectively with moderate rates in nonpolar solvents (Scheme 19.5).^{59,60}

A spectacular organocatalytic α -hydroxylation of ketones and aldehydes was reported using amino acids and chiral pyrrolidines as organocatalysts.⁶¹ The α -hydroxylation of cyclohexanone proceeds with moderate enantioselectivities (e.e. 56% for L-alanine) in the presence of several acyclic amino acids in DMSO with direct formation of the alcohols.⁶² A one-pot enantioselective route to acyclic 1,2-diols from aliphatic aldehydes (*e.g.* **22**) using singlet oxygen conditions and reduction of the intermediary α -hydroxy aldehydes (*e.g.* **23**) with sodium borohydride results in up to 92% e.e.⁶³ Several aspects of these reports are unusual, among them the formation of the hydroxyl carbonyl products in the absence of reducing agents (Scheme 19.6).

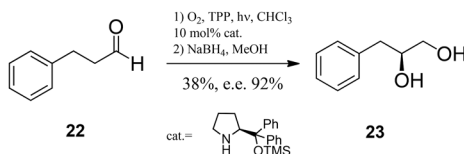
Arylacrylic esters are useful substrates for numerous reactions, *e.g.* the enantioselective synthesis of 2-arylpropionic acids like flurbiprofen.⁶⁴ Due to this role, Jeon *et al.* developed an efficient synthesis for the preparation of 2-arylacrylic esters based on commercially available aryl methyl ketones. The methyl enol ethers **24**, generated by Wittig reaction of aryl methyl ketones, react in a singlet oxygen ene reactions with methylene blue as sensitizer in



Scheme 19.4. Ene regioselectivity.



Scheme 19.5. Allylic alcohols as substrates.



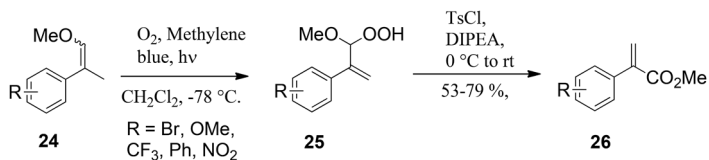
Scheme 19.6. Enantioselective α -oxygenation.

CH_2Cl_2 at -78°C to the allylic hydroperoxides **25**. Tosylation and elimination in a one-pot reaction leads to the desired 2-arylacrylic esters **26** in yields up to 79% (Scheme 19.7).⁶⁵

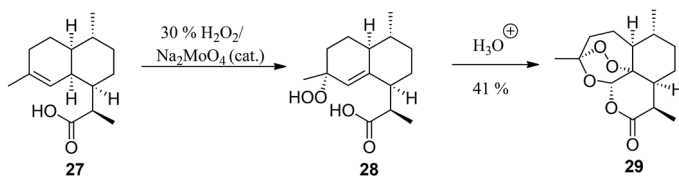
Artemisinin (Art) is, due to its outstanding antimalaria activity, a favorite synthetic target and many semi- and total syntheses have been reported.⁶⁶ One elegant semisynthesis of artemisinin involves the intermediate dihydroartemisinin (DHA).⁶⁷ In a singlet oxygen ene reaction under standard photo-oxygenation conditions the hydroperoxide was formed and acid-catalyzed ring closure leads to artemisinin with 25% overall yield.⁶⁸ A drawback in this synthesis route is the photo-oxidation step because special equipment is needed for large-scale synthesis. This problem was solved by chemical generation of singlet oxygen *via* disproportionation of H_2O_2 in the presence of catalytic amounts of Na_2MoO_4 leading to the hydroperoxide **28** after 12–14 h reaction time. By subsequent acid-catalyzed ring closure, **29** was isolated in 41% overall yield. By using the $\text{Na}_2\text{MoO}_4/\text{H}_2\text{O}_2$ protocol *Chen et al.* developed a facile, efficient and scalable synthesis of artemisinin with no need for special reaction equipment (Scheme 19.8).⁶⁹

1,2,4-Trioxepanes, the next homologs of 1,2,4-trioxanes, can be prepared in four steps from cyclopropyl methyl ketones. A two-step transformation involving Grignard reaction followed by acid-catalyzed rearrangement of the alcohol leads to homoallylic alcohols.⁷⁰ The homoallylic alcohols **30** underwent a highly regioselective photo-oxygenation reaction with singlet oxygen to yield the γ -hydroxyhydroperoxides **31**. Acid-catalyzed peroxyacetalization with different ketones resulted in the 1,2,4-trioxepanes **32**. With this method δ -hydroxy-hydroperoxides and 1,2,4-trioxocanes can be synthesized in gram-scale quantities.⁷¹ The 1,2,4-trioxepane/trioxocanes were also screened for antimalarial activity (Scheme 19.9).^{72,73}

Mayrargue *et al.* investigated the preparation of analogs of the antimalarial terpene peroxides cardamom, a 1,2-dioxepane-containing natural product.⁷⁴



Scheme 19.7. Synthesis of arylacrylic esters.

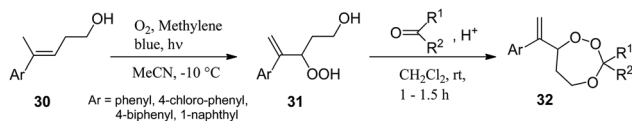


Scheme 19.8. Artemisinin synthesis.

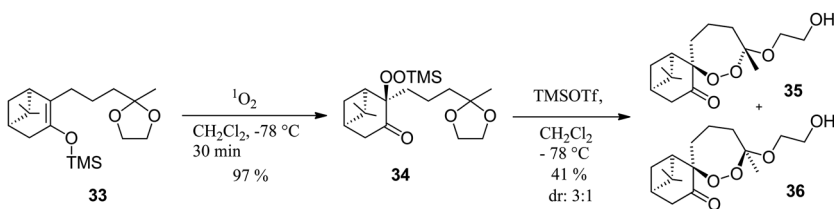
The silylenolether **33**, generated from bromoethylmethyldioxolane, was photo-oxygenated with singlet oxygen to give the α -keto silylperoxide **34** in quantitative yields and with excellent diastereoselectivity. Cyclization of **34** with catalytic amount of TMSOTf leads to the diastereoisomeric 1,2-dioxepanes **35** and **36** in a 3 : 1 diastereomeric ratio (Scheme 19.10).⁷⁵

For oxyfunctionalization of complex substrates the singlet oxygen ene reaction is one of the most important synthetic methods.⁷⁶ It is used in the synthesis of deoxycarbaheptopyranoses⁷⁷ or 4,6-dideoxyfuranoses⁷⁸ and other natural products like Withanolide A, a pharmacologically important steroid.⁷⁹ A representative example is the synthesis of the steroid glaucogenin D **39**, which shows potential antiviral activity.⁸⁰ The first chemical synthesis was reported by Gui *et al.* from a hirundigenin-type intermediate in 12 steps with an overall yield of 6.8%. A key step in this synthesis is the ene reaction of the hirundigenin-type intermediate **37** that resulted in the alkoxy hydroperoxide **38** as a single diastereomer in quantitative yield (Scheme 19.11).⁸¹

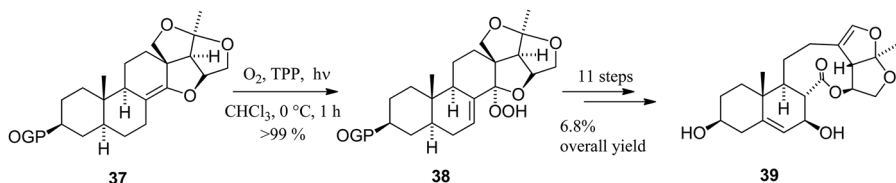
A new and facile method for the synthesis of benzo[*c*]xanthenes, biologically and pharmacologically interesting and active compounds,⁸² offers the one-pot reaction reported by Zheng *et al.* In Scheme 19.12 the proposed mechanism for the formation of benzo[*c*]xanthenes from 2-benzylidene-1-tetralones **40** is shown. After photochemical oxa-6 π electrocyclization, the



Scheme 19.9. Synthesis of trioxepanes.



Scheme 19.10. Antimalarial peroxides.

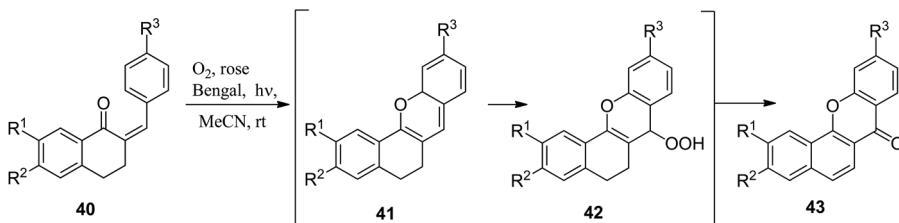


Scheme 19.11. Synthesis of glaucogenin D.

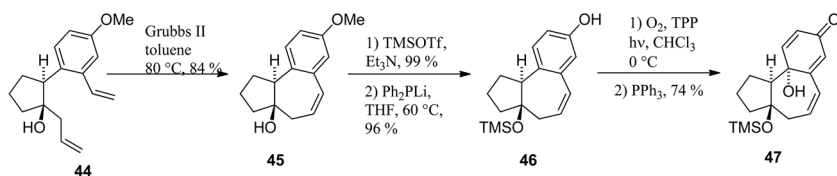
hydroperoxide **42** was efficiently generated by a singlet oxygen ene reaction. The best yields in the ene reaction were achieved with rose bengal as sensitizer and acetonitrile as solvent. Dehydration of the hydroperoxide and aromatization led to the desired product **43** in yields up to 50%.⁸³

Para-Substituted phenols can be converted into the corresponding hydroperoxy-cyclohexadienones under typical singlet oxygen ene conditions.^{84,85} Li *et al.* used this approach for the synthesis of highly functionalized tricyclic frameworks of tigliane and daphnane diterpenoids. Based on three simple building blocks, an aryl bromide, cyclopentene oxide and allylmagnesium bromide, *via* the diene **44** and the metathesis product **45**, the trimethylsilyl ether **46** was synthesized in six steps and 64% overall yield. The key step in this approach is the dearomatization by singlet oxygen reaction to give **47**. Reduction of the hydroperoxy-cyclohexadienone with triphenylphosphine delivered a quinol product as single diastereoisomer (Scheme 19.13).⁸⁶

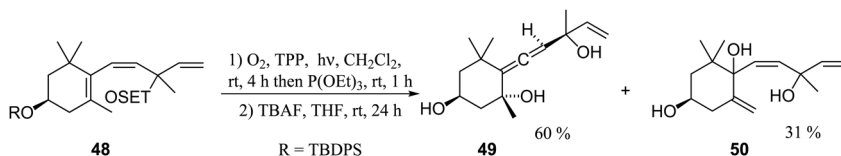
An unusual singlet oxygen ene reaction involves the transfer of a vinylic hydrogen as a synthetic approach to allenylic derivatives carotenoids and was reported by Katsumura *et al.* They discovered, that from the twisted diene **48**, singlet oxygen preferentially abstracts the vinyl hydrogen in competition with an allylic hydrogen. The allene triol **49** was formed in 60% yield together with the regular singlet oxygen ene product **50** in 31% yield (Scheme 19.14).⁸⁷



Scheme 19.12. Synthesis of benzo[c]xanthenes.



Scheme 19.13. Route to daphnane diterpenoids.



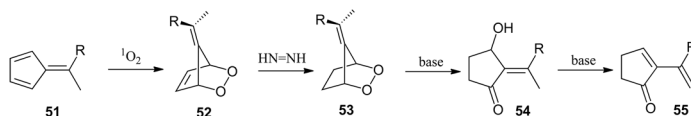
Scheme 19.14. Allenylic alcohols by ene reaction.

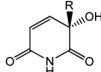
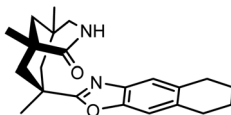
19.4. Applications of the [4+2]-Cycloaddition: Endoperoxide Formation

Since the first report on ergosterol photo-oxygenation,⁸⁸ numerous examples of Diels–Alder-type additions of singlet oxygen were reported. Simple acyclic 1,3-dienes show competing reactivity between ene and 4 + 2-cycloaddition.⁸⁹ Mechanistically, asynchronous concerted and two-step processes have been postulated. As with monoalkenes, the reversible formation of an exciplex possibly precedes product formation. This exciplex collapses to a perepoxide, a 1,4-diradical, or a 1,4-zwitterion. Alternatively, the formation of the [4+2] cycloadduct may take place in a concerted fashion without the involvement of an intermediate. Semiempirical calculations suggested that a stable perepoxide intermediate for the butadiene/singlet oxygen reaction is feasible.⁹⁰ Orbital correlations characterize the [4+2] cycloaddition reaction of singlet oxygen with 1,3-dienes as a stepwise process.⁹¹ The role of charge-transfer (CT) interactions in these reactions and a detailed discussion of the various theoretical aspects is given by Yamaguchi.⁹² Sevin and McKee⁹³ have computed the reaction of singlet oxygen with 1,3-cyclohexadiene at the B3LYP76-31G(d) and CASPT2 levels. They found a stepwise diradical pathway, in which a diradical is formed in the first step with an activation barrier of 6.5 kcal mol⁻¹. The second transition structure has a lower energy than the diradical intermediate and for this reason the reaction could also be considered as a nonsynchronous concerted reaction. Bobrowski *et al.*⁹⁴ studied the 1,4-cycloaddition of singlet oxygen with both *s-cis*-1,3-butadiene and benzene by CAS MCSCF/CAS MCQDPT2 *ab initio* methods. They propose a diradical intermediate on the lowest-energy pathway (MCQDPT2/6-31G(d)) of the reaction of singlet oxygen with *s-cis*-butadiene to the endoperoxide. The stereoselectivity of addition of the singlet oxygen to 1,3-dienes was originally investigated by Rigaudy.⁹⁵ For diastereomeric 2,4-hexadienes, singlet oxygen-induced *E/Z* isomerization was determined at the stage of an intermediate biradical.⁹⁶

The singlet oxygen 4 + 2-cycloaddition to pentafulvenes **51**⁹⁷ with subsequent di-imine reduction delivers saturated bicyclic endoperoxide **53** with an exocyclic double bond.⁹⁸ These adducts are easily base isomerized to 2-vinyl-2-cyclopentenones **55** by a sequence of Kornblum–De La Mare rearrangement⁹⁹ and base-catalyzed (DBU) dehydration.¹⁰⁰ A prerequisite for this reaction is an acidic CH group in the allylic position. This reaction sequence stops at the stage of the hydroxyl enone **54** in the presence of triethylamine as the weaker base. Further thermal rearrangement processes of the saturated bicyclic endoperoxides were published by the same group (Scheme 19.15) (Table 19.1).¹⁰¹

The singlet oxygen 4 + 2-cycloaddition to furans is a remarkably selective process and outpaces in numerous cases all other possible oxygenation sites.^{102,103} Due to the substrate flexibility and the wide occurrence of furans, furan derivatives and furan-derived products in natural product chemistry,

**Scheme 19.15.** Photo-oxygenation of pentafulvenes.**Table 19.1.** Enantioselectivities and yields of template-mediated photo-oxygenation/Kornblum–De La Mare rearrangement.

	R	Yield [%]	e.e. [%]	
in the presence of:	H	99	90	
	Me	96	<5	
	Et	99	86	
	Benzyl	73	69	
	–CH ₂ OEt	30	79	
	–CH ₂ OBn	38	85	

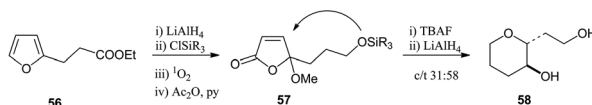
104

the singlet oxygen approach for furan modification has had an impressive renaissance in the last decades.^{104,105} The primary products of this reaction constitute secondary ozonides of cyclobutadienes and as a consequence of this strained structures are highly reactive.^{106,107} Numerous solvents and also ionic liquid solutions can be used that often react with the primary products, *e.g.* alcohols or water.¹⁰⁸ Furthermore, this reaction is indicative for singlet oxygen because other photoinitiated oxygen atom-transfer reactions lead to the corresponding mono-epoxides. This is exemplified for a 2,3-annulated furan that leads to three different products types under singlet oxygen conditions and under photoinduced oxygen-transfer conditions using manganese porphyrine catalysts.¹⁰⁹ Depending on the oxidation stage of the metal porphyrins, diverse modes of oxygen transfer can occur leading to activation of electrophilic *vs.* nucleophilic sites.¹¹⁰

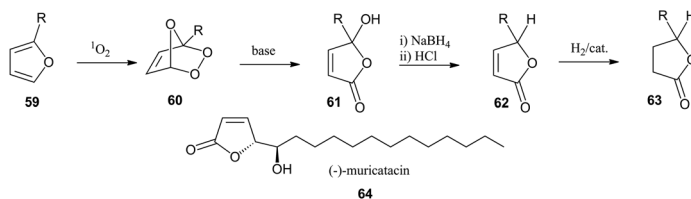
In the presence of alcohols or when alcohols are used as solvents, nucleophilic opening of the peroxide bridge leads to 2-alkoxy-5-hydroperoxy-2,5-dihydrofurans. If tertiary hydroperoxides are formed (*i.e.* monosubstituted furans as **56** are applied), reaction with acetic anhydride in the presence of a base leads to δ -alkoxy-butenolides **57**.¹¹¹ These Michael systems are useful substrates for intramolecular nucleophilic addition with the formation of new carbacycles,¹¹² oxacycles,¹¹³ or thiacycles.¹¹⁴ This is exemplified for the formation of a substituted tetrahydropyran **58** from a monosubstituted furan **56**.¹¹⁵ The butenolide is synthesized from the commercial β -furfuryl-propionic acid by reduction to the corresponding alcohol, silylation, photo-oxygenation in methanol and dehydration. After deprotection of the hydroxyl function, Michael addition and reduction delivered the tetrahydropyran **58** (Scheme 19.16).

When the α -carbon of the furan is unsubstituted, photo-oxygenation of **59** via its ozonide **60** in the presence of a base leads to the corresponding γ -hydroxybutenolides **61** by Kornblum–De La Mare rearrangement, *e.g.* from 3-monosubstituted furans.^{116,117} From methanol solutions, furan and 2-methylfuran result in the two important building blocks γ -methoxybutenolide and γ -methyl- γ -methoxybutenolide,¹¹⁸ useful starting materials for natural-product syntheses.¹¹⁹ Hydride reduction of the ketone carbonyl group of the ene-1,4-dione, that is in equilibrium with the butenolide and recyclization leads to the stable butenolides **62**, as exemplified in the synthesis of the natural γ -lactone *muricatacin* **64**.¹²⁰ A similar approach is reported for the synthesis of the diastereoisomeric butenolides *cladospolides*.¹²¹ From butenolides **62**, butyrolactones **63** are likewise accessible (Scheme 19.17).

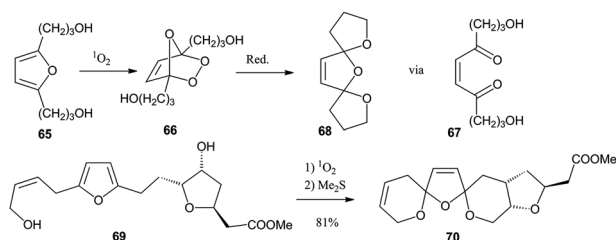
Furan photo-oxygenation with subsequent reduction of the primarily formed endoperoxide **66** is a clean route to 2-ene-1,4-diones **67**, *i.e.* bis-Michael systems, that can react with numerous nucleophiles. By nucleophilic hydroxyl group addition, the 2,5-dihydrofuran ring system can be reformed and bis-spiro ketals **68** are available from 2,5-hydroxyalkylated furans as substrates **65**.^{122,123} This principle was applied in the multistep approach to the unsymmetric furan precursor **69** that, after singlet oxygen reaction and reduction with dimethylsulfide, delivered the ABCD ring skeleton **70** of the azaspiracids family with high, yet undetermined, diastereoselectivity (Scheme 19.18).¹²⁴



Scheme 19.16. Furan photo-oxygenation to tetrahydropyranes.



Scheme 19.17. Synthesis of butenolides.



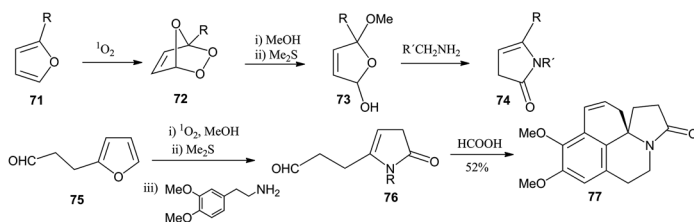
Scheme 19.18. Synthesis of bis-spiro ketals.

Singlet oxygen addition to 2-monosubstituted furans **71** in methanol followed by reduction delivers methoxy lactols **73** that can be reacted with primary amines to give enamides **74** by a substitution/dehydration sequence.¹²⁵ These compounds constitute excellent building blocks for many applications, *e.g.* the intramolecular acyl iminium ion formation and ring annulation. By this multistep and high atom-economic process, the tetracyclic framework of erythrina alkaloids **77** is available *via* the furfurylcarbaldehyde **75** and its intermediary enamide **76** (Scheme 19.19).¹²⁶

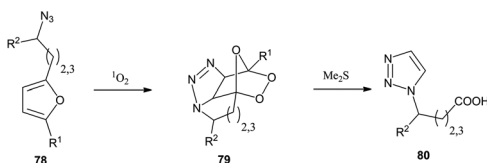
The photo-oxygenation of 2-azidoalkylfurans **78** with appropriate distance between the functional groups allows the intramolecular 1,3-dipolar trapping of the double bond in the initial formed secondary ozonide. These adducts **79** when reduced with dimethylsulfide resulted in 1,2,3-triazoles **80** by a tetrahydrofuran-ring opening and degradation of one of the initial furan ring carbons (Scheme 19.20).¹²⁷

Derivatives of tropone are key structural elements in several biologically active natural products and useful tools in synthetic chemistry, particularly benzotropolones. A new approach to benzotropolones is the photo-oxygenation of benzotropones by singlet oxygen.¹²⁸ Güney *et al.* examined the photo-oxygenation of benzotropones with nitrile groups, due to the important role in bioactive molecules. The photo-oxygenation of compound **81** leads to the expected endoperoxide **82**. This endoperoxide rearranges on silica gel to the acetal product **83**. The photo-oxygenation of the parent compound **84** failed because of the non-planarity of the seven-membered ring in the benzotropone (Scheme 19.21).¹²⁹

Euryfurans are excellent starting materials for the synthesis of important polyfunctional biologically active drimanic compounds. Vlad *et al.* reported the results of regio- and stereoselective semisynthesis of (+)-6-ketoeuryfuran **93** and the photo-oxygenation to (+)-6-ketowinterine **94** starting from (+)-larixyl acetate **86**. The starting material was selected because the bicyclic carbon skeleton is similar to the structure of drimanes.¹³⁰ The reaction sequence



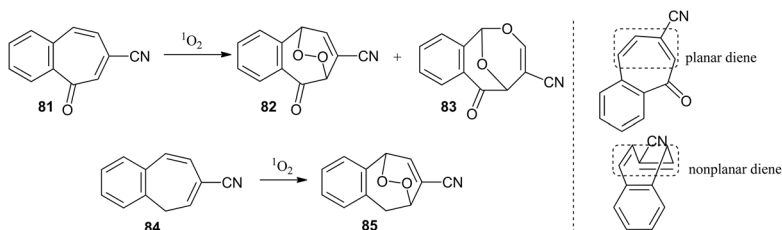
Scheme 19.19. Enamides from furan photo-oxygenation.



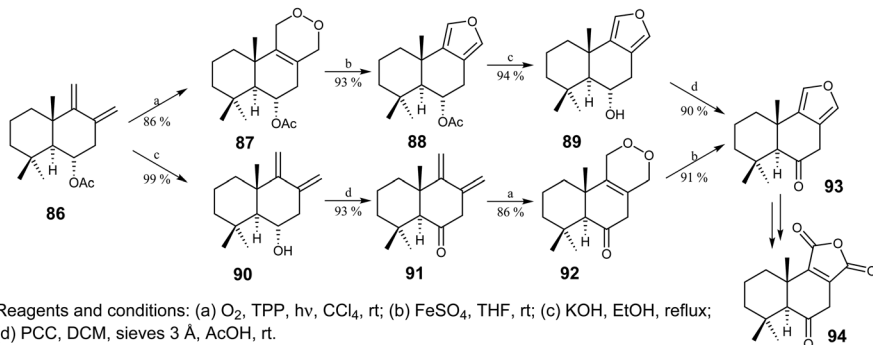
Scheme 19.20. Triazoles from furan photo-oxygenation.

was investigated following two different concepts of step chronology: initial endoperoxide **87** formation or late photo-oxygenation to the keto endoperoxide **92** (Scheme 19.22).

Inspired by natural products, Fattorusso *et al.* described the synthesis, the evaluation of *in vitro* antiplasmodial activity, and the SAR studies for a series of endoperoxide antimalarials based on the plakortin scaffold.¹³¹ The endoperoxide functionality plays a crucial role in the antimalarial activity of these molecules.¹³² The modified Knoevenagel product **95** reacts with singlet oxygen to the endoperoxide **96**. A series of derivatives of this endoperoxide **97–100** were prepared by reaction on the ester and the endocyclic double bond functionalities (Scheme 19.23).¹³¹

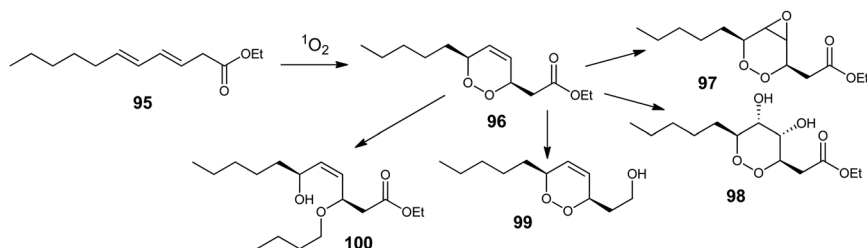


Scheme 19.21. Photo-oxygenation of benzotropones.



Reagents and conditions: (a) O_2 , TPP, hv, CCl_4 , rt; (b) FeSO_4 , THF, rt; (c) KOH, EtOH, reflux; (d) PCC, DCM, sieves 3 Å, AcOH, rt.

Scheme 19.22. Synthesis of euryfurans.



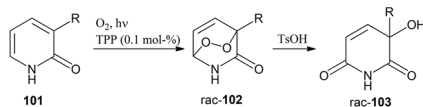
Scheme 19.23. Monocyclic endoperoxide antimalarials.

Kanaoka *et al.* studied the type-II photo-oxygenation of substituted 2-pyridones **101**.¹³³ In the presence of the chiral complexing agent **104** the 3-substituted 2-pyridones **102** were converted enantioselectively into the respective 3-hydroxypyridine-2,6-diones **103** by template-mediated type-II photo-oxygenation and subsequent acid-catalyzed rearrangement. Enantioselectivities have been consistently above 65% e.e. up to 90% e.e.¹³⁴ The synthesis of a 6-hydroxylated epimer of shikimic acid from natural shikimic acid was conducted by a sequence of protecting steps, dehydration to a 1,3-cyclohexadiene and photosensitized singlet oxygen cycloaddition reaction (Scheme 19.24).¹³⁵

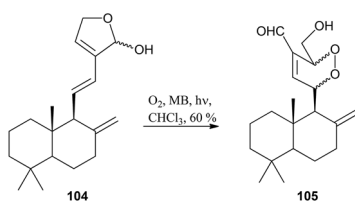
Margaros and Vassilikogiannakis established short and efficient syntheses of chinensines A-E that allowed the determination of the relative stereochemistry of the endoperoxide rings of chinensines D and E and the structurally related members.¹³⁶ These molecules are of interest because of their analgetic and cytotoxic properties. The endoperoxide **105** is available by [4+2]-cycloaddition of the diene **104** (Scheme 19.25).

Photo-oxygenation reactions involving the benzene ring are limited to electron-donating-substituted¹³⁷ and structurally strained benzene derivatives.¹³⁸ Maeda *et al.* described the synthesis of DNA-like fluorescent oligomers composed of alkynyl β -D-ribofuranosides. They observed the instability of these molecules when exposed to ambient indoor lighting and so they examined the photo-oxygenation of similar perylene derivatives **106**. Condensed polyaromatic compounds have low triplet energies and they can serve to sensitize singlet oxygen generation without the need for external photosensitizers.¹³⁹ The self-sensitized photo-oxygenation of **106** delivers regioisomeric endoperoxides **107**, **108** and subsequently regioisomeric dihydroperylene diones **109** and **110**, respectively (Scheme 19.26).

Peroxide-containing lactones showed activity against the malaria parasite *Plasmodium falciparum* and they are also potent cytotoxic substrates. Starting from the aromatic derivatives **111**, Aldeco-Pérez *et al.* established the



Scheme 19.24. Photo-oxygenation of 2-pyridones.



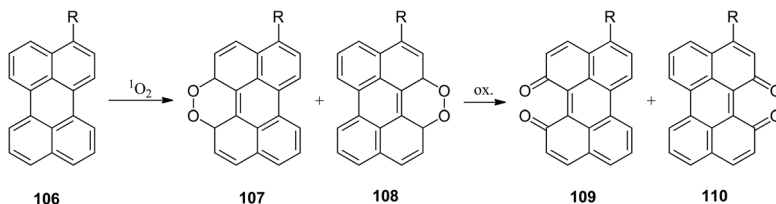
Scheme 19.25. Synthesis of chinensines.

synthesis of lactone endoperoxides **112** and determined the cytotoxicity of these molecules, which is dependent on the nature of the substituent on the remaining double bond (Scheme 19.27).¹⁴⁰

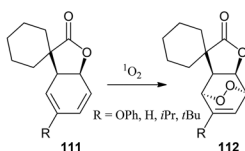
Ryanodine is a natural product isolated from plants of the genus *Ryania* and shows strong interaction with calcium-channel receptors.¹⁴¹ Wood *et al.* synthesized the *trans*-fused bridgehead-oxygenated [4.3.0]-bicyclic product similar to that in ryanodine. The reaction sequence starts with oxidation of a phenol derivative **113** with [bis(trifluoroacetoxyl)iodo]-benzene followed by singlet oxygen addition to get the respective endoperoxide **115** with desired relative stereochemistry (Scheme 19.28).¹⁴²

The sesquiterpenes isolated from the exotic plant species *Illicium merrillianum* and *Illicium floridanum* were named tashironins. They have hemiketal functionality on a caged tetracyclic scaffold based on the biogenetically interesting *allo*-cedrane skeleton. Metha and Maity examined the [4+2]-cycloaddition of diene **116** and subsequent Zn-AcOH reduction to get the *cis*-1,4-enediol **118** with a similar substructure as tashironin (Scheme 19.29).¹⁴³

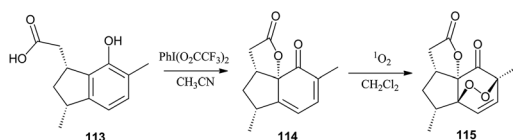
Zhou *et al.* published the enantioselective synthesis of sesquiterpenoid dihydroagarofuran derivative **122** starting from (–)-carvone **119**. These molecules are of interest because of their biological properties such as



Scheme 19.26. Photo-oxygenation of alkynylperylene.



Scheme 19.27. Synthesis of endoperoxide lactones.



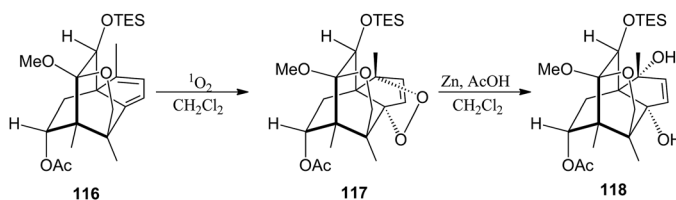
Scheme 19.28. Synthesis of ryanodine derivatives.

anti-HIV¹⁴⁴ and anticancer activity.¹⁴⁵ One step of the synthetic pathway is the [4+2]-cycloaddition of the diene **120**. In this case singlet oxygen was generated from the low-temperature adduct of triphenylphosphite and ozone (Scheme 19.30).¹⁴⁶

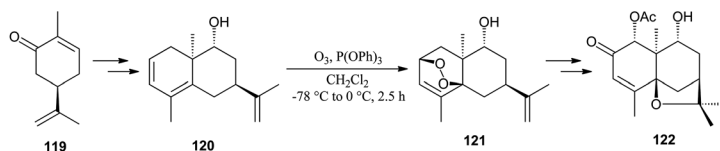
19.5. Applications of Tandem Reactions and Miscellaneous

Para-Substituted phenols **123** were converted in the corresponding 6-hydroperoxy-cyclohexa-1,4-diene-3-ones **124** with singlet oxygen,¹⁴⁷ probably *via* an initial electron-transfer step. Secondary reactions often involve the reduction of the hydroperoxy group and, in case of 4-functionalized side chains, the formation of spiro-oxacycles **125**. The same products that are formed from the singlet oxygen reaction of 4-alkylated phenols can also be obtained from the reaction of the caroate reagent Oxone®¹⁴⁸ with these substrates.^{149–151} The *in situ* reduction of the primary hydroperoxides with thiosulfate is a versatile route to quinols. Formally, these reactions resemble ene reactions, however, electron-transfer steps are highly probable leading to superoxide radical anion and the phenoxy radical that combine to the products (Scheme 19.31).

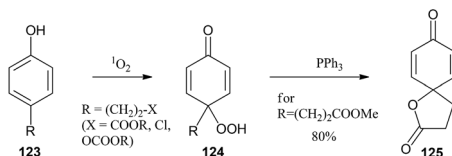
The singlet oxygen reaction of tetrahydronaphthalene **126**, photocatalyzed by TPP, resulted in the formation of a tricyclic product **128**. The first



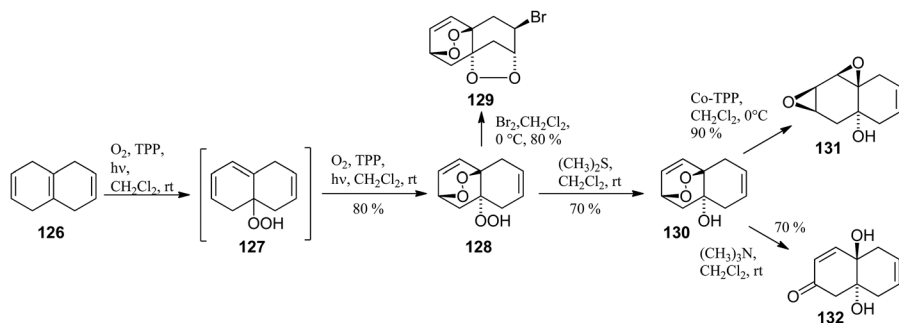
Scheme 19.29. Synthesis of tashironin analogs.



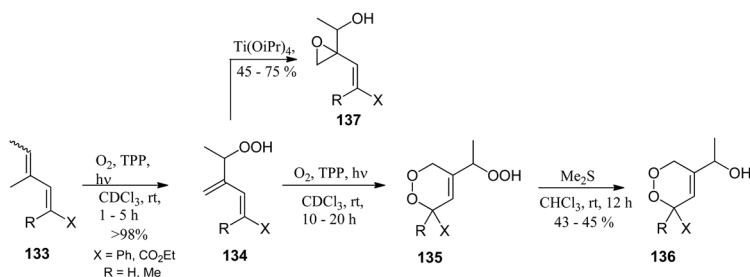
Scheme 19.30. Synthesis of dihydroagarofuran.



Scheme 19.31. Phenol singlet oxygenation with caroate.



Scheme 19.32. Tandem reactions with tetrahydronaphthalene.

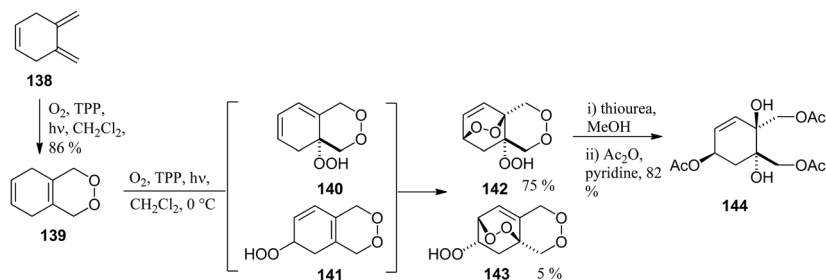


Scheme 19.33. Tandem reactions with acyclic polyene esters.

singlet oxygen is an ene process followed by another singlet oxygen [4+2] cycloaddition to furnish the endoperoxide **128** as sole product in 2 h and 80% yield. Bromination of the hydroperoxide–endoperoxide offers a new and stereospecific synthesis for bis-endoperoxide **129**.¹⁵² Reduction of the **128** hydroperoxide–endoperoxide gives the alcohol **130** and further chemical transformation with trimethylamine, triphenylphosphine or a Co-porphyrin catalyst open the way to a variety of polyhydroxylated decalin derivatives, e.g. **131** and **132** (Scheme 19.32).¹⁵³

Griesbeck *et al.* investigated the photo-oxygenation of 1,3-dienes¹⁵⁴ and 1,3,5-dienes,¹⁵⁵ to find new routes to highly functionalized cyclic peroxides. During these investigations a vinylogous gem effect was observed for the γ,δ -dimethylated and α,γ,δ -trimethylated substrates. In a highly selective first step of the tandem reaction, the vinylogous gem effect favors the formation of hydroperoxide–diene **134** from substrates **133**, which react in a second singlet oxygen reaction to hydroperoxy-1,2-dioxenes **135**. The tandem products as well as the primary addition products are useful substrates for further oxyfunctionalization such as reduction to **136** or oxygen-transfer epoxidation to give **137** (Scheme 19.33).¹⁵⁶

Balci *et al.* reported the first example in which three equivalents of singlet oxygen are involved in a cascade oxygenation process. Photo-oxygenation of 4,5-dimethylenecyclohex-1-ene **138** in methylene chloride at 0 °C with TPP



Scheme 19.34. Triple tandem singlet oxygen reaction.

as sensitizer led to the endoperoxide **139** in only 25 min and 86% yield. The resulting endoperoxide takes up another equivalent of singlet oxygen in an ene reaction and results in two regioisomeric hydroperoxides **140**, **141**, that rapidly underwent the third and last [4+2] cycloaddition reaction. The final products of the cascade photo-oxygenation reaction are two regioisomeric tricyclic bis-endoperoxide **142** and **143**, respectively, which are excellent precursors for the synthesis of carbasugars (*e.g.* **144**) (Scheme 19.34).¹⁵⁷

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Chapter 20

Reactions of Singlet Oxygen with Nucleic Acids

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20.1. Introduction

Singlet oxygen ($^1\text{O}_2$) in the $^1\Delta_g$ state ($E = 22.4 \text{ kcal mol}^{-1}$) is one of the main reactive oxygen species (ROS) which with hydroxyl radical ($\cdot\text{OH}$) and one-electron oxidants such as carbonate anion radical ($\text{CO}_3^{\cdot-}$), potassium bromate (KBrO_3) and type-I photosensitizers are capable to oxidize DNA and RNA.^{1–5} Unlike $\cdot\text{OH}$ that efficiently reacts with almost all biomolecules, $^1\text{O}_2$ exhibits a much higher selectivity since only a few cellular constituents that possess double bonds rich in electrons are the targets of this ROS. Three main reactions have been identified including [2+4] Diels–Alder cycloaddition, [2+2] cycloaddition and the ene reaction that lead to endoperoxides, dioxetanes and allylic hydroperoxides, respectively. A common source of $^1\text{O}_2$ is represented by endogenous and exogenous photosensitizers operating through the type-II mechanism that involves triplet energy transfer from mostly UVA-excited molecules to molecular oxygen.^{6,7} In addition $^1\text{O}_2$ has been found to be generated upon UVB-irradiation of thymine, uracil, cytosine, adenine⁸ and 2'-deoxyguanosine 5'-monophosphate^{9,10} in aerated aqueous solutions. It was also recently shown that recombination of the guanine radical cation, the one-electron oxidation product of guanine, with superoxide anion radical ($\text{O}_2^{\cdot-}$) gives rise to $^1\text{O}_2$.¹¹ Several thionucleobases including 4-thiouracil,¹² 2-thiothymine derivatives^{13–15} and 6-thioguanine^{16–18} are efficient generators of $^1\text{O}_2$ upon UVA excitation. There are also sources of $^1\text{O}_2$ that do not require photoactivation. One relevant example is the biochemical system that in neutrophil phagosome consists of myeloperoxidase, hydrogen peroxide (H_2O_2) and hypochlorous (HOCl).^{19–21} It is well documented that excited-state carbonyls are able to generate $^1\text{O}_2$ in the dark.²² Evidence has been provided at least in model studies that recombination of peroxy radicals derived from either oxidized pyrimidine bases²³ or lipid peroxides^{24,25} led to the formation of $^1\text{O}_2$. This was rationalized in terms of Russell mechanism that involves transient formation of tetroxides.²⁶ It was recently assessed on the basis of laser-based photosensitized methods that involved generation of $^1\text{O}_2$ and its monitoring through the characteristic 1275 nm phosphorescence emission that the intracellular lifetime of $^1\text{O}_2$ is within the range ~ 0.5 to a few μs in single cells incubated with nondeuterated water,²⁷ although there are still uncertainties on the accuracy of the measurements.²⁸ This, which is much larger than earlier estimates, should allow diffusion to some extent of $^1\text{O}_2$ within the cells before being trapped by reactive biomolecules including the guanine moiety of nucleic acids,^{29,30} unsaturated lipids^{31,32} and a few amino acids such as tryptophan, tyrosine, histidine, methionine and cysteine.^{33–35}

Emphasis has been placed in the chapter on the oxidation reactions mediated by $^1\text{O}_2$ with the highly susceptible guanine nucleobase in both isolated and cellular nucleic acids. This includes discussion of the comprehensive mechanisms concerning the formation of primary and secondary guanine oxidation products and their measurement in the DNA of cells and human skin. In the last part of the chapter several relevant examples of $^1\text{O}_2$ -mediated

oxidation reaction of cellular DNA involving endogenous and exogenous photosensitizers are provided and critically reviewed.

20.2. Oxidation of Model Compounds

The highest reactivity of singlet oxygen $^1\text{O}_2$ towards guanine among canonical nucleobases was demonstrated through the comparison of the measured total quenching rate constant (k_t), the sum of chemical quenching (k_r) and physical quenching (k_q) from the decay rate of $^1\text{O}_2$ luminescence at 1270 nm. Thus, the k_t values of the purine and pyrimidine bases of suitably protected DNA nucleosides dissolved in 1,1,2-trichlorotrifluoroethane were found to decrease in the following order: guanine ($3.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) $\gg$ cytosine ($0.058 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) $>$ adenine ($0.018 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) $>$ uracil ($0.011 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) $>$ thymine ($0.0069 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$).³⁶ Similar k_t quenching values were observed for guanine in acetone- d_6 and 2'-deoxyguanosine 5'-monophosphate in aqueous solution that were, respectively, $6.33 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $5.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.^{37,38} A similar value ($k_t = 5.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) was determined for 2'-deoxyguanosine (dGuo) in aqueous solutions using 3,3'-(naphthylidene) dipropionate endoperoxide (NDPO₂) as the clean source of $^1\text{O}_2$.³⁹ It was further confirmed that guanine is the only reactive nucleobase upon exposure of isolated DNA in aqueous solutions to a chemical source of $^1\text{O}_2$.⁴⁰

20.2.1. Guanine Nucleosides and Nucleotides

Early studies on the mechanism of $^1\text{O}_2$ oxidation of the guanine moiety of model compounds in aqueous solutions have involved dGuo⁴¹⁻⁴³ and thymidyl-(3',5')-2'-deoxyguanosine⁴⁴ using phthalocyanine, methylene blue (MB) and naphthalocyanine as predominant type-II photosensitizers with UVA radiation as the source of light. The two main photo-oxidation dGuo products, initially misassigned as the 4R* and 4S* diastereomers of 4-hydroxy-8-oxo-4,8-dihydro-2'-deoxyguanosine (4-OH-8-oxodGuo), were proposed to be the 4R and 4S deoxyribonucleoside derivatives of spiroiminodihydantoin (dSp). This was achieved indirectly from the characterization of the two related oxidized ribonucleosides generated by MB photosensitization of guanosine (Guo).⁴⁵ Interestingly, the latter modified nucleosides had been found a few months earlier to be the main one-electron oxidation products of 8-oxo-7,8-dihydroguanosine (8-oxoGuo) in neutral and slightly alkaline aqueous solutions.^{46,47} More direct support for the spirocyclic connectivity of dSp was provided by highly informative SELINQUATE ¹³C NMR measurements.⁴⁸ Furthermore, the absolute configuration of the two dSp diastereomers that was initially proposed by considering NOE measurements has recently received confirmation from detailed experimental and computational studies of their biochemical and spectroscopic features.^{49,50} As a further piece of information, guanidinohydantoin ribonucleosides are generated at the expense of Sp in acidic aqueous solutions of Guo exposed

to $^1\text{O}_2$.⁵¹ Subsequently, 4R* and 4S* diastereomers of 4-OH-8-oxodGuo have been identified, however as minor $^1\text{O}_2$ oxidation products of dGuo.⁵² Importantly, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) is also generated in, however, about 3- to 4-fold lower yield than dSp when only primary reactions of $^1\text{O}_2$ with dGuo are concerned. The formation of 8-oxodGuo plateaus when the level of oxidized reaches about 0.6% due to the occurrence of secondary oxidation pathways that predominantly give rise to dSp, as further discussed below. Additionally, 2,5-diimino-4-[(2-deoxy- β -D-*erythro*-pentofuranosyl)amino]-2H,5H-imidazole (dD) has been identified in a subsequent study as a minor product of MB photosensitized degradation of dGuo.⁵³ However, it remains to be demonstrated that dD arises from a decomposition pathway triggered by $^1\text{O}_2$ and not from one-electron oxidation reaction since MB is also able to operate through type-I photosensitization mechanism.⁵⁴

The formation of $^1\text{O}_2$ oxidation products of the guanine moiety of related nucleosides and nucleotides may be rationalized in terms of initial Diels-Alder [4+2] cycloaddition involving the 7,8 and 4,5-ethylenic bonds of the imidazole ring (Figure 20.1). This has received support from the ^{13}C NMR measurement of guanine 4,8-endoperoxides produced by type-II photosensitization of 2',3',5'-O-(*tert*-butyldimethylsilyl)-8-methylguanosine in aerated CD_2Cl_2 .⁵⁵ Opening of the 4,8-endoperoxide is likely to generate 8-hydroperoxy-2'-deoxyguanosine (8-OOH-dGuo) that upon subsequent reduction gives rise to the 8-enol tautomer of 8-oxodGuo that is in dynamic equilibrium with the more-stable 6,8-diketo isomer.⁵⁶ Competitive dehydration of 8-OOH leads to the formation of a quinonoid-like intermediate that is highly susceptible to nucleophilic addition.^{51,57,58} Addition of a water molecule across the 5,7-double bond give rises to unstable 5-hydroxy-8-oxo-7,8-dihydro-2'-deoxyguanosine (5-OH-8-oxodGuo) that can further undergo dehydration or hydration to form dSp or dGh, respectively.

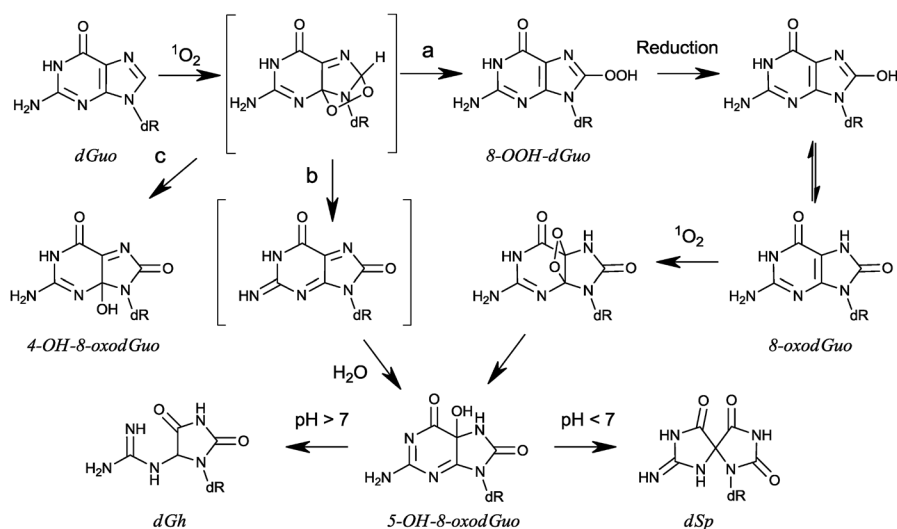


Figure 20.1. Main oxidation pathways of 2'-deoxyguanosine (pathways a-c) and isolated DNA (pathway a) by $^1\text{O}_2$.

(5-OH-8-oxodGuo) that rearranges into either dSp *via* an acyl shift or dGh depending on the pH of the aqueous solutions.^{51,57,58} It was found that dSp diastereomers may also be formed *via* secondary $^1\text{O}_2$ oxidation of 8-oxodGuo, as further discussed below. This was inferred from mechanistic studies involving ^{18}O -labeling experiments^{58,59} and low-temperature NMR measurements.⁵⁶

20.2.2. Thiobases

UVA excitation of 4-thiouracil (4-TUra), a component of tRNA,⁶⁰ leads to the generation of $^1\text{O}_2$ ^{18,61} through efficient quenching of the triplet excited state by O_2 with a rate constant of $\sim 3.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ as inferred from time-resolved infrared spectroscopy measurements.⁶¹ Released $^1\text{O}_2$ is able to subsequently react with 4-TUra, giving rise to predominant fluorescent uracil-6-sulfonate (Ura^{SO_3}) together with lower amounts of uracil (Ura). Mechanistic insights into the two competitive pathways were gained from time-dependent density-functional theory (TD-DFT) calculations at the B3LYP/6-311+G(d,p) level. The formation of Ura^{SO_3} is rationalized in terms of two successive $^1\text{O}_2$ oxidation reactions giving rise to transient uracil-4-sulfinate. This is as an efficient reaction with a low energy barrier of only $1.9 \text{ kcal mol}^{-1}$ that involves a peroxy intermediate (Ura^{SOOH}).⁶¹

Incubation of cells with thiopurines azathioprine, 6-mercaptopurine, and 6-thioguanine (6-TGua) led to accumulation of strongly UVA absorbing 6-thioguanine into nuclear DNA.⁶² Exposure of 6-TGua as the free nucleobase or embedded into oligonucleotides led to the generation of a fluorescent nucleobase that was identified as guanine-6-sulfonate (Gua^{SO_3}).^{16,63} In addition, guanine-6-sulfinate (Gua^{SO_2}) was found to be efficiently generated as a stable primary photoproduct⁶⁴ that may undergo further oxidation to Gua^{SO_3} by a second molecule of $^1\text{O}_2$, as shown in a recent theoretical study.⁶⁵ The TD-DFT calculations thus applied are in favor of a peroxy intermediate (G^{SOOH}) similarly to the $^1\text{O}_2$ oxidation of 4-TUra as the first step, giving rise in an efficient and low energy barrier reaction to Gua^{SO_2} (Figure 20.2) and not to guanine-6-sulfenate (Gua^{SO}) as initially proposed.^{16,64} The rate constant of the reaction of $^1\text{O}_2$ with 6-TGua leading to Gua^{SO_3} is high with a value of $4.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ that was calculated using a fluorometric method.⁶⁵ It may be pointed out that a competitive reaction leads to the formation of guanine as a minor photoproduct, in agreement with the unfavorable energy profile of the reaction.⁶⁵ The reported photochemical and photophysical

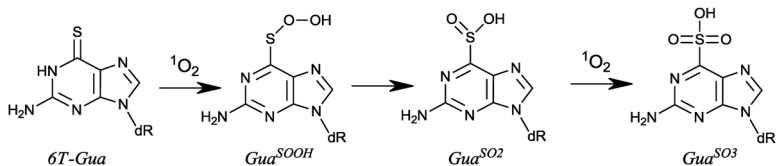


Figure 20.2. $^1\text{O}_2$ oxidation of 6-thioguanine.

features of 6-TGua provide molecular explanations for the high observed phototoxicity of thiopurine derivatives associated with their therapeutic utilization.

20.2.3. 8-Oxo-7,8-Dihydroguanine as Part of Nucleosides and Oligonucleotides

The elevated susceptibility of (8-oxoGuo) to $^1\text{O}_2$ that has been estimated to be about two orders of magnitude higher than that of Guo³⁸ explains the efficient consumption of 8-oxodGuo as soon it is produced by type-II photosensitizers in aerated aqueous solutions of dGuo.⁴¹ Early experiments aimed at identifying the main $^1\text{O}_2$ oxidation products of 8-oxodGuo have involved MB as the photosensitizer.^{66,67} The main oxidized nucleosides include 1-(2-deoxy- β -D-*erythro*-pentofuranosyl)cyanuric acid together with, however, in smaller amounts of 2,2-diamino-4-[2-deoxy- β -D-*erythro*-pentofuranosyl]amino]-5-(2*H*)-oxazolone (dZ) and 2-amino-5-[(2-deoxy- β -D-*erythro*-pentofuranosyl)amino]-4*H*-imidazol-4-one (dIz), its hydrolytically unstable precursor.⁶⁷ However, excited MB, while being a fair generator of $^1\text{O}_2$, is also able to act with 8-oxodGuo as a one-electron oxidant⁵⁴ giving rise to highly reactive radical intermediates.^{68,69} Therefore, this together with the presence of $\text{O}_2^{\cdot-}$ as a side product of the oxidation of the MB radical anion by O_2 is likely to interfere with the $^1\text{O}_2$ -degradation pathways of 8-oxodGuo. This received indirect support when thermolabile endoperoxide from the naphthalene derivative *N,N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide (or 3,3-(1,4-epidioxynaphthalene-1,4-diylbis-[*N*-(2,3-dihydroxypropyl)propanamide) (DHPN), denoted DHPNO₂ or DHPN¹⁸O₂⁷⁰ was used as the clean source of unlabeled or [¹⁸O]-enriched $^1\text{O}_2$.⁵⁹ The predominant oxidation products were measured using the accurate and quantitative high-performance liquid chromatography coupled to the electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) method. They include, in addition to previously identified dZ and dIz, the two diastereomers of dSp and oxidized guanidino-hydantoin 2'-deoxyribonucleoside (dGh^{ox}), at the exclusion, however, of the cyanuric acid nucleoside.⁵⁹ The reported findings are rationalized in terms of initial formation of diastereomeric dioxetanes through [2+2] cycloaddition of $^1\text{O}_2$ across the 4,5-ethylenic bond of 8-oxodGuo which after opening of the O–O bond gives rise to 5-hydroperoxy-8-oxo-5,7-dihydro-2'-deoxyguanosine (5-OOH-8-oxodGuo).⁵⁹ Reduction of the peroxide function and subsequent purine-ring rearrangement of the resulting alcohol constitute the key steps leading to the formation of the pair of dSp diastereomers. Support for the proposed mechanism is provided by ¹³C NMR and 2-D HMBC measurements of 4,5-endoperoxides⁷¹ and related 5-hydroperoxide⁷² upon $^1\text{O}_2$ oxidation of the TBDMS derivative of 8-oxoGuo in organic solvent at low temperature. This also includes the unambiguous assignment, through extensive NMR measurements, of 5-hydroxy-8-oxo-5,7-dihydroguanosine that is formed upon mild reduction of the 5-hydroperoxide precursor by dimethylsulfide at $-40\text{ }^\circ\text{C}$.⁷²

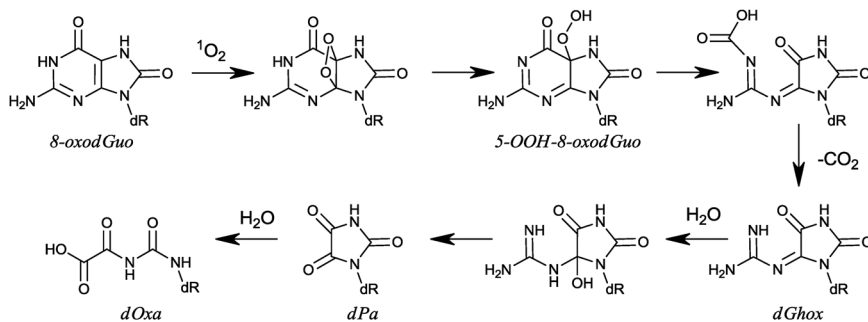


Figure 20.3. Secondary $^1O_2$ oxidation of 8-oxo-7,8-dihydroguanine containing oligonucleotide.

A different degradation pattern was observed following $^1O_2$ oxidation of 8-oxodGuo containing single-stranded 15-mer oligodeoxyribonucleotide since the 2'-deoxyribonucleoside derivative of oxaluric acid (dOxa) is the predominant degradation product without detectable amounts of dSp.⁷³ However, the initial step of the oxidative degradation pathway of 8-oxodGuo appears to be similar to what described for the isolated nucleoside that involves initial formation of 4,5-dioxetanes followed by rearrangement into 5-OOH-8-oxodGuo (Figure 20.3). The main difference concerns the subsequent step that is characterized by the absence of reduction of the latter peroxide, which was only a minor process in the oxidation of isolated 8-oxodGuo.⁵⁹ Rearrangement of the 5-hydroperoxide that involves opening of the pyrimidine ring at the C5–C6 bond through α -ketohydroperoxide cleavage followed by a decarboxylation reaction gives rise to relatively stable dGh^{ox}. In a subsequent step dGh^{ox} is converted into parabanic acid 2'-deoxyribonucleoside derivative (dPa) through nucleophilic addition of a water molecule prior to being hydrolyzed into dOxa.⁷³ Interestingly, $^1O_2$ oxidation of 8-methoxy-2'-deoxyguanosine, that mimics the configuration of the 8-enol tautomer of 8-oxodGuo gives rise to dIz as the main degradation product instead of dOxa when the modified nucleoside is embedded into a trideoxynucleotide.⁷⁴

20.3. Oxidation of Isolated Nucleic Acids

Most of the investigations aimed at determining the $^1O_2$ -mediated oxidative pathways of isolated nucleic acids have focused on DNA with, however, a few studies devoted to RNA.

20.3.1. DNA

Earlier attempts to characterize oxidizing effects of $^1O_2$ on isolated DNA and more precisely the guanine moiety have involved the use of MB as the photosensitizer.^{75,76} The search for 8-oxodGuo was facilitated by the development

of a suitable HPLC electrochemical detection method operating in the oxidative mode that became available a few years earlier.⁷⁷ Thus, it was found that 8-oxodGuo is predominantly generated in calf thymus DNA, whereas low amounts of DNA strand breaks were also detected.^{75,76} The formation of DNA nicks is likely to be accounted for by secondary $^1\text{O}_2$ oxidation of initially generated 8-oxodGuo that gives rise to labile lesions such as dOz. Subsequently, dSp diastereomers were found to be produced with a yield that was lower by two orders of magnitude than that of 8-oxodGuo upon MB photosensitization of calf thymus DNA.⁴² It may also be pointed out that 8-oxodGuo is the main degradation product of type-II photosensitized oxidation of DNA by either *meso*-tetra(4-*N*-methylpyridyl)porphyrin (*p*-TMPyP) or *meso*-tetra(4-sulfatophenyl)porphyrin (TSPP), a nonintercalating DNA photoactive agent.⁷⁸ This was confirmed on the basis of accurate HPLC-ESI-MS/MS measurements that 8-oxodGuo is the almost exclusive $^1\text{O}_2$ oxidation product of isolated DNA upon incubation with DHPNO₂ endoperoxide, at least when the level of 8-oxodGuo is lower than 40 lesions per 10⁶ normal bases.⁴⁰ This is in agreement with recent HPLC-ESI-MS/MS measurements of dSp and dOz whose relative frequencies with respect to 8-oxodGuo were less than 10⁻² in calf thymus DNA upon rose bengal (RB) photosensitization.⁷⁹ The yield of dGh was even 10-fold lower than that of dSp and dOz. These consistent findings clearly show that in contrast to what was observed for isolated dGuo, the dehydration pathway of 8-OOHdGuo giving rise to reactive quinonoid intermediate is, at best, a minor process in the $^1\text{O}_2$ oxidation of native DNA. Therefore, the overwhelming formation of 8-oxodGuo may be rationalized in terms of predominant reduction of 8-OOHdGuo, the rearrangement product of 4,8-guanine endoperoxides. Consequently the formation of guanine adducts that were proposed to arise from the nucleophilic addition of free amino groups of spermine to the quinonoid-like species derived from guanosine⁸⁰ is an unlikely reaction to occur in DNA. It may also be pointed out that the formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) that was proposed to be a $^1\text{O}_2$ oxidation product of DNA⁸¹ has been ruled out.⁴⁰

20.3.2. RNA

RNA oxidation reactions triggered by $^1\text{O}_2$ have received little attention so far. As a striking result MB and RB photosensitized formation of 8-oxoGuo has been measured in the RNA that was isolated from calf liver.⁸² In another investigation a comparative study of the formation of 8-oxoGua was performed in single-stranded DNA, native DNA and RNA upon photosensitization by MB, TMPyP and TSPP.⁷⁸ It was found that RNA and ssDNA showed a 5-fold higher susceptibility to $^1\text{O}_2$ oxidation when TSPP was used as the photosensitizer.⁷⁸ 8-OxoGuo has been also shown to be generated in the RNA of Q β bacteriophage upon photosensitization by either RB⁸² or MB.⁸²⁻⁸⁴

20.4. Oxidation of Cellular DNA by $^1\text{O}_2$

Numerous attempts were made during the last two decades for measuring oxidatively generated damage to cellular DNA under various oxidative conditions where $^1\text{O}_2$ was at least partly involved. Two main topics are covered in this section. One deals with the careful delineation of the oxidative pathways induced by a clean source of $^1\text{O}_2$ to cellular DNA. Information is also provided on the contribution of $^1\text{O}_2$ to the photodynamic effects mediated by UVA irradiation. However, the available data on the formation of 8-oxodGuo triggered by exogenous photosensitizers which were reported in several review articles^{7,85,86} are not further discussed in the present chapter.

20.4.1. Chemical Source of $^1\text{O}_2$ as the Oxidant

Unambiguous information on the oxidation reactions mediated by $^1\text{O}_2$ on nuclear DNA has been gained from the use of DHPNO₂ as a clean source of $^1\text{O}_2$. Thus, the release of $^1\text{O}_2$ from the thermolabile endoperoxide precursor within the cells is accompanied by the selective oxidation of guanine residues in the DNA of THP1 monocytes.⁸⁷ This was inferred from the measurement of 8-oxodGuo using an accurate and specific HPLC-ESI-MS/MS method operating in the multiple reaction monitoring (MRM) mode. It was further confirmed using [$^{18}\text{O}_2$]-labeled DHPNO₂ that the increase in 8-oxodGuo formation arose from released $^1\text{O}_2$ and not as the result of the induction of oxidative stress possibly triggered by the incubation of the cells with the endoperoxide. Interestingly, [^{18}O]-labeled 8-oxodGuo containing cellular DNA was used as an internal standard for assessing the suitability of several extraction methods to minimize the occurrence of adventitious oxidation of the guanine moieties during DNA work-up.⁸⁸ Another relevant piece of information concerning the oxidizing features of $^1\text{O}_2$ was gained upon incubation of human monocytes with DHPNO₂. Thus, it was clearly demonstrated using the alkaline comet assay as the sensitive analytical tool that $^1\text{O}_2$ generation did not increase significantly the levels of DNA strand breaks and/or alkali-labile sites.⁸⁹

20.4.2. UVA Radiation

It is well documented, as critically discussed in several review articles,^{85,86,90} that UVA irradiation of mammalian cells led to the photosensitized formation of 8-oxodGuo that, however, is much lower in terms of efficiency than the generation of cyclobutane pyrimidine dimers through direct excitation of the pyrimidine bases in human fibroblasts and keratinocytes.^{91,92} A similar observation was made in the DNA of human skin explants that were exposed to UVA radiation.⁹³ It may be remembered that in any case the detection of 8-oxodGuo provides relevant information on the mechanism(s) involved in the formation of this ubiquitous oxidized 2'-deoxyribonucleoside that can

be generated by $^1\text{O}_2$, $\cdot\text{OH}$ and one-electron oxidants.^{1,30} This explains why an appropriate strategy was devised to gain mechanistic insights in the UVA-sensitized formation of 8-oxodGuo through the measurement of strand breaks and oxidation products of pyrimidine and purine bases induced by UVA radiation and gamma rays.⁹⁴ For this purpose three classes of DNA lesions including oxidized nucleobases revealed as DNA repair glycosylase-sensitive sites were detected and quantified in human monocytes using the alkaline comet assay. Strong differences were observed in the relative formation of the three main classes of DNA damage from the comparison of the degradation effects of UVA light and ionizing radiation. Thus UVA irradiation led to the overwhelming formation of purine lesions over pyrimidine modifications and strand breaks that are formed in about 6-fold lower yields.⁹⁴ This contrasts with a more balanced distribution of radiation-induced classes of damage to DNA whose formation is mostly accounted for by $\cdot\text{OH}$ -mediated reactions.^{1,30} Thus, strand breaks and or alkali-labile sites, modified purine bases and oxidized pyrimidine bases are generated in a relative ratio 2.5:1:1.⁹⁴ Similar observations were made in UVA-irradiated mammalian cells through the measurement of several classes of DNA damage including strand breaks, endonuclease III- and formamidopyrimidine DNA glycosylase-sensitive sites using the alkaline elution technique.^{7,95,96} It was thus concluded that the formation of 8-oxodGuo in the DNA of UVA-irradiated human monocytes accounted for about 80% to $^1\text{O}_2$ oxidation as the result of type-II photosensitization mechanism.⁹⁴ The remaining 20% that led to formation of DNA strand breaks and oxidized pyrimidine bases are likely to be explained in terms of $\cdot\text{OH}$ -mediated degradation pathways. This involves initial generation of $\text{O}_2^{\cdot-}$, followed by its spontaneous or enzymatic dismutation into H_2O_2 that is reduced into $\cdot\text{OH}$ according to the Fenton reaction. It remains to assess what is the relative contribution of the reactions mediated by $^1\text{O}_2$ and $\cdot\text{OH}$ in the enhanced UVA-photosensitized formation of 8-oxodGuo in human melanocytes⁹⁷ with respect to monocytes.

20.5. Conclusions

Abundant and relevant information is available on the $^1\text{O}_2$ oxidation reactions of nucleic acids that included detailed mechanistic studies on model compounds and the measurement of oxidatively generated damage to DNA in cells and human skin. As a striking finding it was found that $^1\text{O}_2$ oxidation of guanine, the exclusive DNA target, gives rise predominantly to 8-oxoGua in cellular DNA. Evidence has been provided that $^1\text{O}_2$ is the main oxidizing species involved in the UVA sensitized formation of 8-oxoGua. It remains to be assessed what is the contribution of $^1\text{O}_2$, the ROS of a type-II photosensitization mechanism, with respect to one-electron oxidation (type-I mechanism) in the UVA-induced formation of 8-oxoGua by exogenous photosensitizers such as methylene blue and 6-thioguanine⁹⁸ that are widely used as therapeutic drugs. It would be of interest in the case of 6-thioguanine, once

incorporated into cellular DNA where it may act as both a type-I and type-II internal photosensitizer to look for the occurrence of secondary oxidation reactions to initially generated 8-oxoGua. Another relevant topic to be further investigated would consist in assessing the reactivity of cellular RNA to $^1\text{O}_2$ with emphasis on the guanine and 2-thiouracil bases.

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Chapter 21

Reactions of Singlet Oxygen with Membrane Lipids: Lipid Hydroperoxide Generation, Translocation, Reductive Turnover, and Signaling Activity

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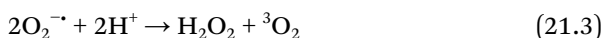
21.1. Introduction

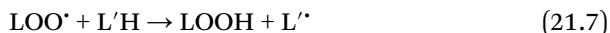
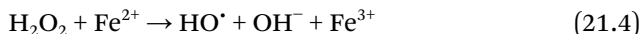
When generated under carefully controlled conditions using applied or metabolically induced sensitizers, singlet oxygen ($^1\Delta_g\text{O}_2$ or $^1\text{O}_2$) can be exploited for therapeutic purposes, the best-known example being photodynamic therapy (PDT) for eradication of various malignant tumors.^{1,2} Considerable attention has been directed to the relative reactivity of different molecular targets with $^1\text{O}_2$ (proteins *vs.* nucleic acids *vs.* membrane lipids) in an effort to better understand how PDT and other photodynamic processes damage and inactivate mammalian cells.² It has been argued that proteins are the major $^1\text{O}_2$ targets in most cells because total protein levels and rate constants for $^1\text{O}_2$ reactivity far exceed those of nucleic acids or unsaturated lipids.³ Although this argument may hold in general, it ignores critical factors such as where the sensitizer may preferentially localize within cells, how O_2 (*i.e.* $^3\text{O}_2$) levels may vary in different subcellular compartments, and $^1\text{O}_2$'s relatively short lifetime, *i.e.* limited diffusion radius.⁴ Many PDT sensitizers (*e.g.* porphyrins, phthalocyanines) are amphiphilic and can partition into membrane lipid compartments, which are typically O_2 rich compared with aqueous compartments. These factors would tend to favor unsaturated lipids as $^1\text{O}_2$ targets even though the reaction rate constants are considerably lower than those of proteins. With this in mind, we will review several aspects of $^1\text{O}_2$ -mediated lipid photo-oxidation, including basic reaction mechanisms, methods for monitoring reactions, use of lipid hydroperoxide intermediates (LOOHs) as mechanistic reporters, intermembrane translocation of LOOHs, one-electron *vs.* two-electron reductive turnover, and role of LOOHs in stress signaling.

21.2. Lipid Photoperoxidation in Biological Membranes

21.2.1. Basic Mechanisms: Singlet Oxygen *vs.* Free-Radical Intermediacy

As in all photodynamic reactions, the primary postexcitation chemistry of lipid photoperoxidation can be classified as either Type I or Type II, the former involving free radicals, *e.g.* those generated by triplet sensitizer-reducing substrate ($^3\text{S}/\text{RH}$) interactions, and the latter typically involving $^1\text{O}_2$ generated by triplet-sensitizer-ground-state oxygen ($^3\text{S}/^3\text{O}_2$) interactions.⁵⁻⁷ In a cell experiencing a Type-I process, RH might be NAD(P)H, ascorbate, glutathione, or some membrane-localized species, but probably not a lipid itself due to insufficient reducing potential.⁶ The process might commence according to eqn (21.1),



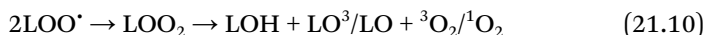


where a substrate radical ($\text{R}^\bullet$) and sensitizer radical anion ($\text{S}^{\bullet-}$) are produced, either of which might induce a lipid peroxidation cascade. In the case of $\text{S}^{\bullet-}$, autoxidation (eqn (21.2)) gives superoxide ($\text{O}_2^{\bullet-}$), which gives rise to hydroxyl radical ($\text{HO}^\bullet$) *via* disproportionation to hydrogen peroxide (H_2O_2), followed by the Fenton reaction (eqn (21.3) and (21.4)). Hydroxyl radical abstracts an allylic hydrogen from an unsaturated lipid (*e.g.* a phospholipid *sn*-2 fatty acyl group), generating a carbon-centered lipid radical (eqn (21.5)), which reacts rapidly with O_2 to give a lipid peroxy radical ($\text{LOO}^\bullet$) (eqn (21.6)). The latter then abstracts a hydrogen from another lipid molecule ($\text{L}'\text{H}$) to initiate free radical-mediated lipid peroxidation, often referred to as chain peroxidation (eqn (21.7)). The mechanism shown (eqn (21.1)–(21.7)) is one of several that could be envisaged for different reactant (${}^3\text{S}/\text{RH}$) pairs and conditions. Each of these free radical-mediated pathways can give rise to a large number of primary and secondary oxidation products, including a variety of aldehydes in the case of polyunsaturated phospholipids.⁸ Thus, Type-I lipid photoperoxidation is relatively complex and can be difficult to characterize with certainty because, among other complications, there may be a background of nonphotodynamic lipid peroxidation (*i.e.* autoxidation) in the system studied, and this may be difficult to control in certain situations. Type-II photoperoxidation is considerably less complex mechanistically than Type I and in general gives far fewer products. The process begins with transfer or energy (94 kJ mol^{-1}) from triplet sensitizer to O_2 , giving ${}^1\text{O}_2$ and ground state sensitizer (eqn (21.8)). The ${}^1\text{O}_2$



then reacts with an unsaturated *sn*-2 fatty acyl group of a phospholipid or at the C-5 or C-6 position of cholesterol to give a hydroperoxide species with the double bond shifted to the allylic position (eqn (21.9)). This is an example of the so-called “ene” reaction of ${}^1\text{O}_2$ with olefins.^{6,9} It is important to note that this is an addition reaction and all three atoms of the $-\text{OOH}$ group derive from ${}^1\text{O}_2$ and the target lipid (eqn (21.9)). This contrasts with Type I (free radical)-generated $-\text{OOH}$, where the two oxygen atoms derive from ${}^3\text{O}_2$ and the hydrogen atom from another lipid (eqn (21.6) and (21.7)). A special feature of ${}^1\text{O}_2$ -derived LOOH s is that in the absence of reductants and catalytic metal ions, they can accumulate linearly with increasing ${}^1\text{O}_2$ concentration, *i.e.* without the lag typically observed in free-radical reactions.¹⁰ Although

purely $^1\text{O}_2$ -induced lipid peroxidation does not involve free radicals, these might arise secondarily as a result of one-electron reduction of $^1\text{O}_2$ -generated LOOHs (see below). On the other hand, free-radical lipid peroxidation might give rise to $^1\text{O}_2$ *via* the Russell mechanism¹¹ in which two peroxy radical intermediates react to produce an unstable tetroxide, which decomposes to an alcohol, a ketone, and oxygen (eqn (21.10)).



The O_2 is either in the triplet ground state or singlet excited state, the ketone being in the triplet excited or ground state, respectively. Although $^1\text{O}_2$ generated *via* the Russell reaction has been demonstrated for reactions in organic solvents and liposomal membranes,¹² there is no direct evidence to date that it can occur in a natural membrane or lipoprotein environment.¹³

21.3. Differentiation Between Type-I and Type-II Photoperoxidation

Classical approaches based on use of free radical scavengers or $^1\text{O}_2$ quenchers and lifetime modulators have been used in attempts to assess whether photodynamic lipid peroxidation occurs *via* the Type-I or Type-II mechanism. In the case of $^1\text{O}_2$, avid interceptors such as azide, histidine, and β -carotene have been employed, and inhibitory effects have been reported.^{5,9} In other cases, inhibition by superoxide dismutase or catalase has been described, implying Type-I chemistry and $\text{O}_2^{\bullet-}$, H_2O_2 , and presumably $\text{HO}^\bullet$ intermediacy. One cannot probe for $^1\text{O}_2$ in analogous fashion because it has no known enzymatic scavengers. Inhibition of a reaction by desferrioxamine, an iron chelator/redox inhibitor, might suggest $\text{HO}^\bullet$ involvement (eqn (21.4)) *via* Type-I chemistry. A Type-I reaction might also be discerned using $\text{HO}^\bullet$ scavengers (*e.g.* mannitol, deoxyribose) or $\text{LO}^\bullet/\text{LOO}^\bullet$ scavengers (*e.g.* butylated hydroxytoluene, α -tocopherol). It is important to point out that in complex systems, competitive kinetic studies with such agents are virtually impossible because the lipid substrates and their concentrations are typically not well defined. Therefore, the identity of peroxidation-inducing ROS is usually based on qualitative information rather than rigorous kinetic analysis. In addition, many of the inhibitors mentioned lack absolute specificity. For example, in addition to intercepting $^1\text{O}_2$, azide and histidine in relatively high concentrations can quench triplet-state sensitizers or bind/inactivate iron and other redox metal ions.^{14,15} Moreover, azide and histidine can also react with $\text{HO}^\bullet$, whereas α -tocopherol, a widely used radical scavenger, can react quite rapidly with $^1\text{O}_2$.⁹ Added to these uncertainties is the fact that $\text{HO}^\bullet$ generated on a membrane may be poorly accessible to hydrophilic $\text{HO}^\bullet$ scavengers and that the latter in high concentrations might distort membrane structure, resulting in nonspecific effects. It is important to point out that purely Type-II lipid peroxidation may turn into a free radical-propagated process if primary ($^1\text{O}_2$ -derived) LOOHs undergo iron-catalyzed one-electron

reduction (see Section 21.4.1). There is solid evidence that this can occur in cellular systems under photo-oxidative stress.¹⁶ For such systems, a reliable $^1\text{O}_2$ trap will inhibit primary LOOH formation, but free radical-traps would inhibit downstream LOOH formation, leading to confusion about Type-II vs. Type-I importance. The possibility of using D_2O to elucidate the mechanism should also be considered. Knowing that $^1\text{O}_2$'s lifetime is increased ~ 15 -fold upon switching from aqueous medium to D_2O ,⁴ one might deduce that Type-II photoperoxidation is occurring if the reaction is accelerated in D_2O . This approach requires that solvent quenching be the major determinant of $^1\text{O}_2$ lifetime and that H_2O be readily exchanged for D_2O at reaction sites. However, since membrane lipids exist in nonaqueous environments, the chance of seeing a significant D_2O effect would be quite low, and not seeing one would not rule out $^1\text{O}_2$ involvement.

21.3.1. Lipid Hydroperoxides as Mechanistic Reporters

A more reliable approach for mechanistic characterization of photosensitized lipid peroxidation would be one based on identification of signature lipid products, *i.e.* those obtained exclusively from $^1\text{O}_2$ or free-radical reactions. The clear advantage of this is that products generated *in situ* are exploited without the need for exogenous probes, which might perturb the system being studied. Select phospholipid and cholesterol hydroperoxide species can be exploited as endogenous mechanistic reporters, although the latter have distinct advantages, as will be discussed. For a common phospholipid such as 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), abstraction of a linoleoyl doubly allylic hydrogen by a strong oxidant such as $\text{HO}^\bullet$ gives an alkyl radical ($\text{L}^\bullet$) with a delocalized free electron (eqn (21.5)), Figure 21.1). This reacts rapidly with O_2 to give two positional peroxy radicals, one at C9 and the other at C13 of the linoleoyl moiety, and each with conjugated double bonds.^{8,10} Hydrogen abstraction from a proximal neighboring lipid gives the corresponding hydroperoxides, 9-OOH and 13-OOH, and a new propagating alkyl radical (Figure 21.1). By contrast, $^1\text{O}_2$ attack on PLPC gives four hydroperoxide species, 9-OOH, 10-OOH, 12-OOH, and 13-OOH, 9- and 13-OOH being conjugated and 10- and 12-OOH being nonconjugated.¹² Thus, identification of the latter two PLPC oxidation products in a photoreaction system would unambiguously demonstrate at least partial $^1\text{O}_2$ involvement. High-sensitivity/specificity LOOH detection is now possible,^{17,19} but direct chromatographic separation of different positional phospholipid hydroperoxides (PLOOHs) such as those described is very difficult, so mechanistic analysis at this molecular level remains elusive. Although fatty acid hydroperoxides liberated by chemical or enzymatic hydrolysis of PLOOHs can be readily resolved by HPLC or HPTLC methods and analyzed for peroxide content,²⁰ peroxide instability and inevitable degradation during hydrolysis and subsequent workup would be problematic. One possible solution would be to reduce peroxides to alcohols prior to hydrolysis, but this would dramatically reduce detection options and sensitivities thereof.

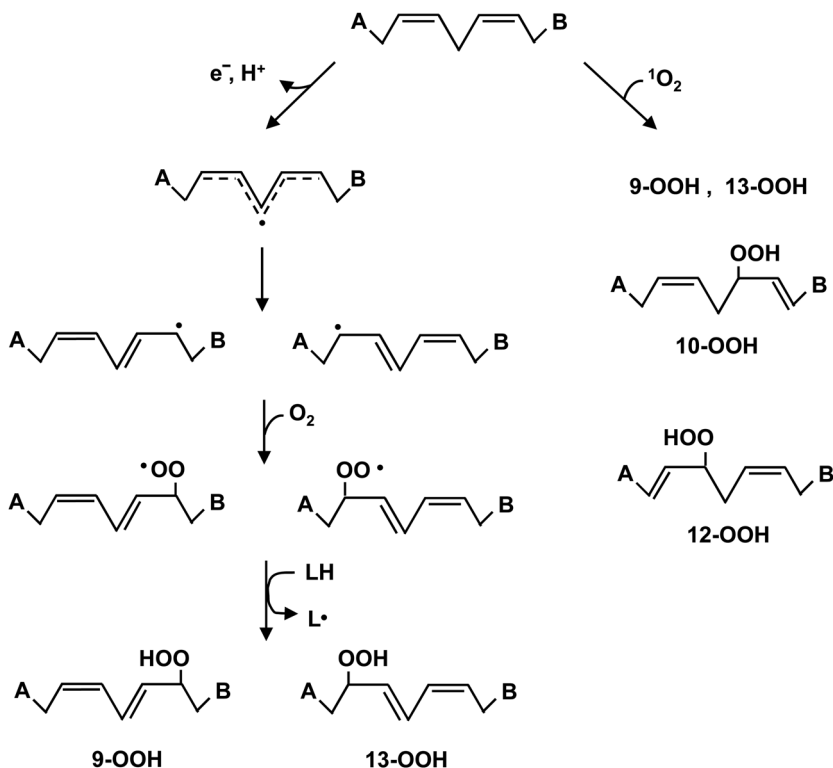


Figure 21.1. Photoperoxidation of a phospholipid *sn*-2 linoleoyl group *via* a Type-I (free radical) or Type-II (singlet oxygen) mechanism. A = $CH_3(CH_2)_3-$; B = $-(CH_2)_6COOH$. Two PLOOH positional isomers are produced *via* the Type-I pathway: 9-OOH and 13-OOH; four *via* the Type-II pathway: 9-OOH, 10-OOH, 12-OOH, and 13-OOH. From: A. W. Girotti, *Photochem. Photobiol.*, 1990, 51, 497–509, with permission.

Most of the problems described in the preceding paragraph can be avoided by employing cholesterol ring hydroperoxides (ChOOHs) as mechanistic indicators. Nonesterified cholesterol (Ch) is found in all mammalian cell membranes, most of it being located in the plasma membrane (~45 mol% of total lipid).²¹ As a monounsaturated lipid, Ch is subject to peroxidation, although not as readily as PLPC and other polyunsaturated phospholipids. There are several advantages of using Ch as an endogenous mechanistic probe; for example, (i) unlike phospholipids, Ch exists as a single molecular species in cell membranes, thereby making product analysis less complex; (ii) ChOOHs and other Ch oxides can be analyzed directly by HPLC or TLC without a requirement for hydrolysis, which could potentially result in loss or other artifacts. In free radical-mediated reactions, including Type-I photoreactions, two major epimeric ChOOHs are observed as early products: 3 β -hydroxycholest-5-ene-7 α -hydroperoxide (7 α -OOH) and 3 β -hydroxycholest-5-ene-7 β -hydroperoxide (7 β -OOH), the latter being more thermodynamically stable.²² These hydroperoxides are typically accompanied by

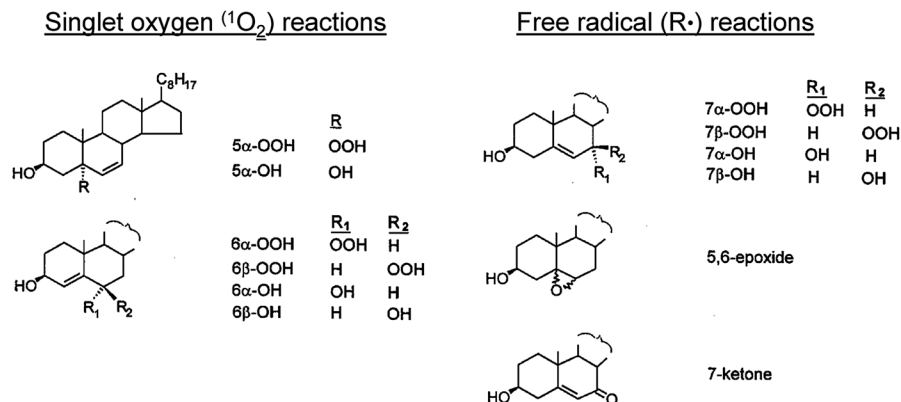


Figure 21.2. Structures of cholesterol oxidation products generated by Type-I (free radical) and Type-II (singlet oxygen) photochemistry.

their hydroxy counterparts, cholest-5-ene-3 β -7 α -diol (7 α -OH) and cholest-5-ene-3 β -7 β -diol (7 β -OH), the 7-ketone (7=O), and the epimeric 5,6-epoxides (5,6-O) (Figure 21.2). 7 α - and 7 β -OOH derive from initial abstraction of a Ch C7 hydrogen by a strong oxidant, which might be either a nonlipid ROS (*e.g.* HO $\cdot$) or a lipid-derived oxidant (*e.g.* LOO $\cdot$). Rapid addition of O $_2$ to the resulting cholesteryl radicals gives peroxy radicals, which abstract hydrogen from other lipids to become 7 α - and 7 β -OOH. Whereas these hydroperoxides may serve as redox-active intermediates (see Section 21.4), 7 α -OH, 7 β -OH, 7=O, and 5,6-O are for the most part redox-inactive end-products of Ch peroxidation. In Type-II photoperoxidation, only three primary ChOOHs are possible: 3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide (5 α -OOH), 3 β -hydroxycholest-4-ene-6 α -hydroperoxide (6 α -OOH), and 3 β -hydroxycholest-4-ene-6 β -hydroperoxide (6 β -OOH), each arising *via* ene-addition of $^1\text{O}_2$ to Ch $^{23-26}$ (Figure 21.2). The initial rate of 5 α -OOH accumulation in a photoperoxidizing membrane usually exceeds that of 6 α -OOH or 6 β -OOH by at least 5-fold ^{28} and among the three ChOOHs, 5 α -OOH always exists at the highest steady-state level. The preponderance of 5 α -OOH is attributed to steric hindrance, which results in less-favorable $^1\text{O}_2$ addition at the C6 than at the C5 position. ^{26} Although $^1\text{O}_2$ does not produce 7 α - or 7 β -OOH directly, these ChOOHs may arise indirectly *via* allylic rearrangement of 5 α -OOH, but this is usually insignificant at low levels of membrane peroxidation. ^{26,27} Conversely, no 5 α -OOH or 6 α /6 β -OOH should be produced by primary Type-I chemistry, although these species might possibly arise from $^1\text{O}_2$ generated by downstream Russell-type disproportionation of peroxy radical intermediates (eqn (21.10)). ^{11,12} Up to now, however, no significant 5 α -OOH has been detected in any cellular system undergoing vigorous lipid-chain peroxidation. ^{13,26} Thus, until proven otherwise, detecting 5 α -OOH in a photo-oxidized cell or tissue sample can be taken as unambiguous evidence that at least some Type-II chemistry has occurred. ^{28} Importantly, 5 α -OOH has been detected in the

skin of rats exposed to pheophorbide a-sensitized photoperoxidation, the first documented evidence for $^1\text{O}_2$ production in a living animal.²⁹ From the discussion thus far, it is clear that $5\alpha\text{-OOH}$ or $6\alpha/6\beta\text{-OOH}$ could serve as a definitive Type-II reporter. Similarly, $7\alpha/7\beta\text{-OOH}$ could serve as Type-I reporters, but in this case some uncertainty may exist because of a small degree of $5\alpha\text{-OOH}$ rearrangement to $7\alpha\text{-OOH}$ and/or chain peroxidation set off by one-electron reduction of $5\alpha\text{-OOH}$ or other primary peroxides. Since most of the Ch in mature mammalian cells exists in the plasma membrane, Ch can serve as a convenient and reliable molecular probe of photodynamic reactions taking place in this compartment. As discussed in the next section, development of high-sensitivity/specificity approaches for analyzing ChOOH reporters has greatly facilitated mechanistic determinations.

21.3.2. Analytical Methods for Lipid Hydroperoxides

A variety of techniques are available for analyzing lipid peroxidation in general and the LOOHs formed in particular. Relatively simple “bulk-type” methods exist, including the classic thiobarbituric acid (TBA) assay for determining aldehyde byproducts of chain peroxidation and the iodometric assay for determining total extractable LOOH. These methods are now often used as preliminaries to more sophisticated GC- and HPLC-based approaches with very high sensitivity and specificity. Quite often, the chromatographic separations are coupled to mass spectrometry for definitive analyte identification or conformation. For cutting-edge LOOH separation, identification, and quantification, two high-performance approaches are noteworthy, one based on chemiluminescence detection (HPLC-CL)¹⁷ and the other on reductive-mode electrochemical detection (HPLC-EC).¹⁸ In the latter category, a breakthrough came with the introduction of reductive EC detection on a renewable mercury drop [HPLC-EC(Hg)], which was developed in the authors’ laboratory.^{18,19} Reversed-phase HPLC-EC(Hg) has proven to be the method of choice for rapid, high-specificity analyses of ChOOHs and PLOOHs from oxidized systems ranging from simple liposomes to mammalian cells. Moreover, this approach is extraordinarily sensitive, the detection limit for $7\alpha/7\beta\text{-OOH}$, for example, being ~ 200 fmol.¹⁹ An example of HPLC-EC(Hg) chromatographic analysis is shown in Figure 21.3.

The data represent POPC/Ch (10 : 8, by mol) liposomes that had been either nonsensitized (upper scan) or sensitized with AlPcS_4 (lower scan) and exposed to a 90 J cm^{-2} light fluence, using a quartz-halogen source. No signals could be detected in the light control, indicating that LOOH content before and after liposome preparation was insignificant. However, four well-resolved ChOOH peaks were observed in the photoperoxidized sample: $5\alpha\text{-OOH}$, $6\beta\text{-OOH}$, $6\alpha\text{-OOH}$, and $7\alpha/7\beta\text{-OOH}$, in order of decreasing peak intensity, $6\beta\text{-OOH}$ being the most hydrophobic (longest retention time). $5\alpha\text{-OOH}$ was the predominant ChOOH photoproduct, representing $\sim 80\%$ of the total, $6\alpha\text{-OOH}$ plus $6\beta\text{-OOH}$ amounting to $\sim 15\%$, and $7\alpha/7\beta\text{-OOH}$ $< 5\%$. The more hydrophobic species at 12–13 min represent at least two POPC hydroperoxides.

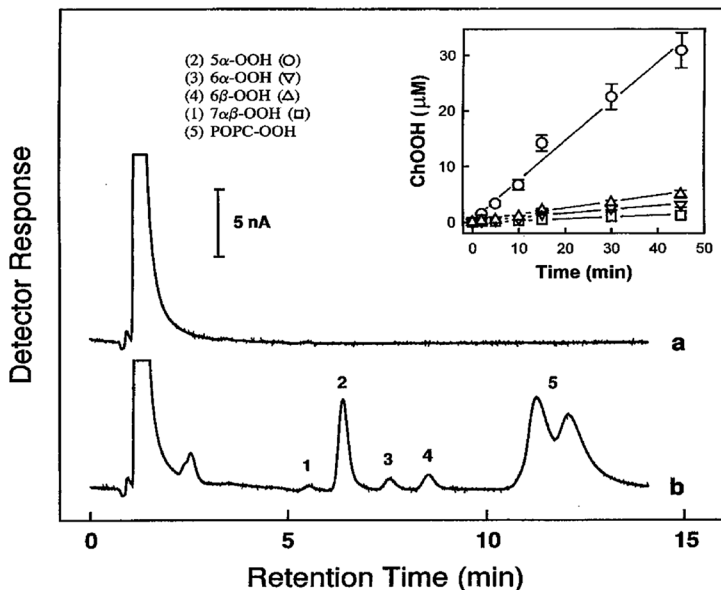


Figure 21.3. HPLC-EC(Hg)-detected LOOHs in lipid extracts from nonsensitized (upper scan) and AlPcS₄-sensitized (lower scan) POPC/Ch (10:8 by mol) unilamellar liposomes that had been exposed to a 90 J cm⁻² light fluence. Peak identities: (1) 7α/7β-OOH; (2) 5α-OOH; (3) 6α-OOH; (4) 6β-OOH; (5) POPC-OOH. Inset: accumulation of 5α-OOH (○), 6α-OOH (Δ), 6β-OOH (▽), and 7α/7β-OOH (□) during irradiation. From: W. Korytowski and A. W. Girotti, *Photochem. Photobiol.*, 1999, **70**, 484–490, with permission.

All of the ChOOH isomers accumulated linearly with increasing irradiation time (Figure 21.3 inset), the 5α-OOH rate being greatest. Preponderance of 5α-OOH over 7α/7β-OOH indicates that a Type-II mechanism applied and that ¹O₂ was the principal oxidant, the 7α/7β-OOH probably arising *via* 5α-OOH rearrangement. By way of comparison, only 7α/7β-OOH, 7α/7β-OH, and 7=O were detected when Ch-containing liposomes were peroxidized using the xanthine/xanthine oxidase system, which generates O₂^{•-}/H₂O₂/HO[•].²⁵ These results would also be expected for purely Type-I lipid photoperoxidation, although the latter remains to be identified experimentally. HPLC-EC(Hg) has also permitted high-sensitivity/specificity detection of ChOOHs in more complex systems such as photo-oxidized tumor cells and lipoproteins.^{30,31}

If specialized methodology such as HPLC-CL or HPLC-EC(Hg) is not available for analyzing PLOOHs and ChOOHs, one could turn to relatively simple normal-phase thin-layer chromatography (TLC). Unfortunately, even when high-performance TLC plates are used with optimal mobile phases, none of the PLOOHs or ChOOHs present in cell or tissue lipid extracts are well resolved from one another, making distinctions (*e.g.* between 5α-OOH and 7α/7β-OOH) extremely difficult. However, when the ChOOHs are reduced by borohydride or triphenylphosphine treatment,

the resulting ChOHs are sufficiently well separated to allow accurate identification.²⁸ Migrating more slowly than ChOOHs, the ChOHs are visualized by spraying the plate with 50% sulfuric acid and warming; 5 α -OH typically migrates well ahead of 7 α -OH and 7 β -OH.^{26,28} TLC analysis for 5 α -OH vs. 7-OH can suffice as an initial screen for $^1\text{O}_2$ vs. free-radical intermediacy, but sensitivity is far below that attained with HPLC-EC(Hg) and quantification is difficult.

21.4. Lipid Hydroperoxide Fates in Biological Systems

21.4.1. Iron-Catalyzed One-Electron Reduction (Damage Expansion)

LOOHs generated under photo-oxidative stress may either be end products or intermediates, depending on local environmental conditions. In systems lacking reducing agents and/or redox metal ions like iron and copper, LOOHs tend to accumulate linearly with stress duration, *e.g.* continuous $^1\text{O}_2$ generation (see Figure 21.3). By their mere presence, these LOOHs can perturb membrane structure/function because their polarity is greater than that of parent lipids. However, if catalytic iron and an appropriate iron reductant gain access to an LOOH, it can undergo one-electron reduction to a highly reactive oxyl radical ($\text{LO}^\bullet$) intermediate (Figure 21.4).^{25,32} The chemistry is analogous to that of the Fenton reaction, where H_2O_2 is reduced to $\text{HO}^\bullet$ by Fe(II) (eqn (21.4)). One-electron reduction to $\text{LO}^\bullet$ by Fe(II) is much more favorable kinetically than one-electron oxidation to $\text{LOO}^\bullet$ by Fe(III) .³³ Iron ligated to a membrane group in close proximity to the reactive LOOH would be more effective in driving the reaction than nonligated iron. A nascent $\text{LO}^\bullet$ may initiate peroxidation chains by abstracting an allylic hydrogen from a proximal unsaturated lipid. Alternatively, a $\text{LO}^\bullet$ may undergo β -scission to an aldehyde and alkyl radical, the latter autoxidizing to a chain-initiating peroxy radical. As another alternative, the $\text{LO}^\bullet$ may undergo rapid rearrangement with O_2 addition to give an initiating epoxyallylic peroxy radical ($\text{OLOO}^\bullet$) (Figure 21.4). The latter mechanism has been demonstrated for simple polyunsaturated fatty acid systems,³⁴ but not for natural membranes. It may also apply for one-electron reduction of ChOOHs to $\text{ChO}^\bullet$ intermediates, but this remains to be investigated. New LOOHs would be generated *via* one-electron reduction of primary species and these would feed into the overall process, thus exacerbating the membrane damage/dysfunction caused by primary LOOHs alone, hence the term “damage expansion” (Figure 21.4). This would most likely be the case for Type-II photoperoxidation, where primary LOOHs are generated by $^1\text{O}_2$ addition without free-radical involvement. The contribution of $\text{LO}^\bullet$ -initiated secondary reactions to photodynamic killing of tumor cells was demonstrated by showing that butylated hydroxytoluene (BHT), a chain-breaking antioxidant, protected against photokilling when introduced immediately after primary LOOH generation.¹⁶

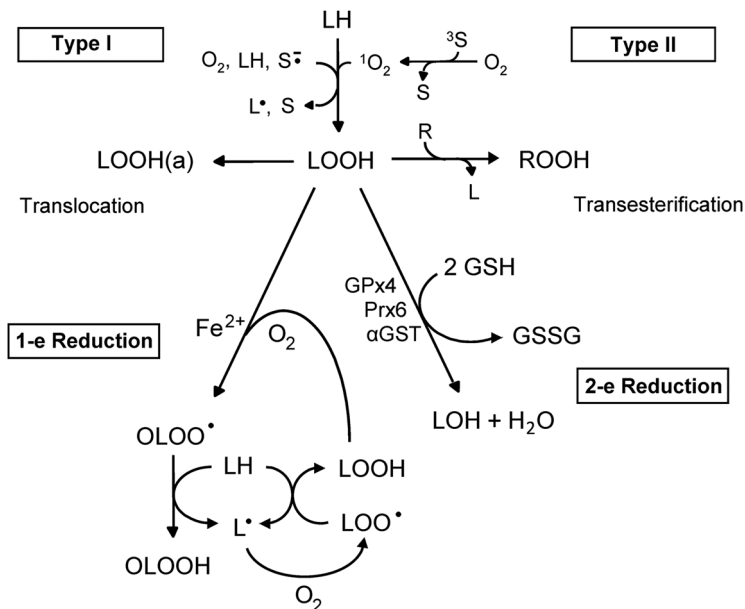


Figure 21.4. Possible fates of LOOHs generated by Type-I or Type-II photoperoxidation. Only some of the possible reactants and products involved in Type-I primary LOOH formation are represented. One-electron reduction results in membrane-damage enhancement, whereas enzyme-catalyzed two-electron reduction results in damage containment. Also represented is transesterification of a peroxidized fatty acyl group to an acceptor lipid (R) and LOOH translocation to an acceptor membrane (a).

In a recent study, the ability of two Ch $^1O_2$ adducts, 5α -OOH and 6β -OOH, to induce membrane-damaging chain peroxidation was compared, with surprising results.³⁵ When unilamellar liposomes composed of POPC, Ch, and 3 mol% of either 5α -OOH or 6β -OOH were incubated with ascorbate and a lipophilic iron chelate, both ChOOHs decayed at the same rate, suggesting equal rates of one-electron reduction. However, when $[^{14}C]$ Ch was used as an endogenous sensor of chain-initiating potency, as monitored by TLC-detected chain products ($[^{14}C]$ ChOX),¹³ 5α -OOH was highly potent, whereas 6β -OOH showed little or no activity.³⁷ Similar contrasting results were obtained when $[^{14}C]$ Ch-loaded leukemia cells were challenged with 5α -OOH vs. 6β -OOH, the former being exceedingly more cytotoxic than the latter.³⁵ Two possible explanations were proposed: one thermodynamic (6β -O $^\bullet$ may be a weak hydrogen abstractor), and the other kinetic (6β -O $^\bullet$ may not be oriented properly for optimal hydrogen abstraction). The latter explanation appears more plausible because 6β -OOH was not totally inactive, but additional supporting evidence is needed. This is the first example of a membrane LOOH being almost inert with regards to initiation of chain peroxidation.

As is well known, photosensitized oxidation can damage and inactivate membrane proteins as well as lipids. Direct attack of $^1O_2$ on proteins with susceptible

amino acid residues is favored when a Type II-sensitizer lies in close proximity. Similarly, attack by a Type-I primary ROS such as H_2O_2 or $\text{HO}^\bullet$ may occur. However, damage induced by postirradiation chain lipid peroxidation may be more important than is recognized by most investigators. A striking example is seen in a study involving merocyanine 540 (MC540)-sensitized photoinactivation of two intrinsic plasma membrane enzymes in leukemia cells: Na^+, K^+ -ATPase and acetylcholinesterase (AChE).³⁶ Chain peroxidation and Na^+, K^+ -ATPase activity loss were both inhibited by the iron chelator desferrioxamine and stimulated by iron supplementation. However, AChE activity loss was completely unaffected by iron manipulation, nor was it affected by the LOOH deactivator ebselen, which strongly inhibited Na^+, K^+ -ATPase loss. The explanation provided was that Na^+, K^+ -ATPase's active site is located within the membrane bilayer, whereas AChE's lies off the membrane surface.^{37,38} Accordingly, chain peroxidation due to one-electron reduction of primary (MC540/ $^1\text{O}_2$) LOOHs appeared to play a key role in Na^+, K^+ -ATPase photoinactivation, but no role at all in AChE photoinactivation, AChE presumably undergoing direct $^1\text{O}_2$ attack.³⁶

21.4.2. Enzyme-Catalyzed Two-Electron Reduction (Damage Control)

Eukaryotic cells are endowed with a rich variety of primary and secondary defenses against lipid peroxidation and other oxidative injuries. Potentially lethal damage can occur if these defenses are overwhelmed and oxidative stress is manifested. The primary defense is usually preventative, *e.g.* (i) scavenging of $\text{O}_2^{\cdot -}$ by superoxide dismutases or H_2O_2 by catalase or glutathione peroxidases, and (ii) sequestration of free redox iron by ferritin. Although $^1\text{O}_2$ has nonenzymatic scavengers, no enzymatic counterparts are known to exist. Consequently, any enzymatic protection against $^1\text{O}_2$ -initiated lipid peroxidation must occur at the secondary or LOOH level. Most of our discussion in this section focuses on attenuation of peroxidative damage *via* the action of LOOH-detoxifying enzymes (Figure 21.4). At least four intracellular enzymes have been implicated in LOOH detoxification: type-1 glutathione peroxidase (GPx1), type-4 glutathione peroxidase (GPx4), type-6 peroxiredoxin (Prx6), and type- α glutathione-S-transferase (α GST).²⁵ GPx1 and GPx4 have been studied more extensively than the others and we will focus on them here. Both of these peroxidases contain an active-site selenocysteine residue, GPx4 being far less abundant in mammalian cells than GPx1. Both enzymes catalyze the two-electron reductive detoxification of hydroperoxides at the expense of glutathione (GSH), but differ in specificity, GPx1 acting on relatively polar peroxides (*e.g.* H_2O_2 and fatty acid hydroperoxides (FAOOHs)) and GPx4 on less-polar species such as PLOOHs and ChOOHs.²⁵ Whereas GPx4 can act directly on membrane-bound PLOOHs and ChOOHs,^{39,40} GPx1 cannot, although the reasons for this difference are not known from the standpoint of rigorous structural biology. For PLOOH reduction, two mechanisms have been proposed: *Excision/Reduction/Repair*, involving PLOOH hydrolysis by phospholipase-A₂ (PLA₂), reduction of liberated FAOOH by GPx1, and lysolipid reacylation at the *sn*-2 position; and (ii) *Reduction/Excision/Repair*,

involving sequential action of GPx4 and PLA₂ followed by reacylation. Most of the evidence to date favors the GPx4-mediated reduction/excision/repair pathway (direct PLOOH reduction *in situ*) as a more effective damage control strategy.⁴¹

Studies in this laboratory revealed that GPx4 can also catalyze the direct two-electron reduction of membrane ChOOHs; GPx4 appears to be unique in this ability.⁴⁰ ChOOHs exhibit a broad range of reactivities with the GPx4/GSH system, the first-order rate constants increasing in the following order: $5\alpha\text{-OOH} \ll 6\alpha\text{-OOH} \approx 7\alpha/7\beta\text{-OOH} < 6\beta\text{-OOH}$. All ChOOH isomers are reduced more slowly than PLOOHs under the same reaction conditions, but the molecular basis for this difference is unknown. This finding is illustrated in Figure 21.5, which represents an experiment in which a natural cellular homogenate was used as the GPx4 source.⁴² Note that severe selenium deficiency (which prevented GPx4 expression) abolished ChOOH detoxification. Note also, that $5\alpha\text{-OOH}$ was the most GPx4-resistant LOOH, consistent with it being the most cytotoxic.⁴²

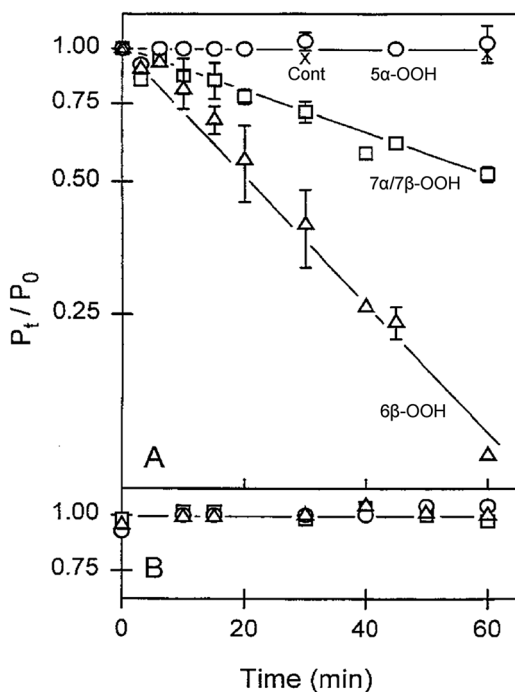


Figure 21.5. Susceptibility of different ChOOHs to GPx4-catalyzed reduction. A lysate of selenium-replete (A) and selenium-starved (B) L1210 cells was incubated with 5 mM GSH and 40 μ M each of $5\alpha\text{-OOH}$, $6\beta\text{-OOH}$, or $7\alpha/7\beta\text{-OOH}$ in redox-inhibited Triton-PBS solution at 37 °C. Lipid extracts from timed samples were analyzed for each ChOOH by HPLC-EC(Hg). P_0 and P_t : ChOOH level at time 0 and time t . From: W. Korytowski, P. G. Geiger and A. W. Girotti, *Biochemistry*, 1996, 35, 8670–8679, with permission.

21.4.3. Spontaneous and Protein-Facilitated Intermembrane Transfer

Studies in the authors' laboratory have revealed that one-electron or two-electron reduction of a nascent LOOH is not necessarily restricted to its membrane of origin, but can extend to neighboring membranes.^{43,44} This is possible in part because LOOHs, being more polar than parental lipids, have greater departure rates from donor membranes. Spontaneous translocation between membranes and also membranes and lipoproteins has been demonstrated.⁴⁵ All LOOHs are capable of translocation, but ChOOHs move from a donor to acceptor membrane much more rapidly than PLOOHs, assuming a single -OOH group per molecule for each lipid type. A model study involving photoperoxidized erythrocyte membrane (ghost) donors and unilamellar liposome acceptors at 10-fold lipid molar excess showed that ChOOHs as a group transferred at least 60-times faster than parent Ch.⁴⁴ Similarly to Ch transfer, ChOOH transfer was found to occur *via* an aqueous diffusion mechanism rather than by donor-acceptor collision, the rate-limiting step being desorption from the donor membrane.^{43,44} Importantly, individual ChOOH isomers were found to transfer with different first-order rate constants, the decreasing order being: $7\alpha/7\beta\text{-OOH} > 5\alpha\text{-OOH} \gg 6\alpha\text{-OOH} > 6\beta\text{-OOH}$, which correlates with their retention time order on reverse-phase HPLC.^{18,19} Experiments with a GPx4-null tumor cell line revealed that cytolethality of three different liposome-supplied ChOOHs decreased in parallel with their rates of transfer uptake by cells, *i.e.* $7\alpha/7\beta\text{-OOH} > 5\alpha\text{-OOH} > 6\beta\text{-OOH}$. However, in GPx4-replete cells, the order switched to $5\alpha\text{-OOH} \gg 7\alpha/7\beta\text{-OOH} > 6\beta\text{-OOH}$, reflecting more importantly, $5\alpha\text{-OOH}$'s poor reactivity with GPx4 (Section 21.4.2). (Note that $6\beta\text{-OOH}$'s minimal toxicity in these experiments is attributed to poor chain initiating potency (Section 21.4.1) rather than slower transfer or faster reduction by GPx4.)

Interest in intermembrane LOOH translocation and its possible role in dissemination of peroxide damage and stress signaling intensified when it was discovered that this process can be accelerated by lipid-transfer proteins.⁴⁶ These proteins are known to play key roles in lipid metabolism, membrane function, and membrane biogenesis. A well-known example is sterol carrier protein-2 (SCP-2), a small intracellular protein that facilitates intermembrane transfer of Ch and other sterols as well as various phospholipids, hence its alternate designation as a nonspecific lipid-transfer protein. Studies in the authors' laboratory showed that naturally occurring as well as recombinant SCP-2 could markedly accelerate LOOH translocation from photoperoxidized erythrocyte ghost membranes to unilamellar liposomes and *vice versa*.⁴⁶ This was the first reported evidence for such an effect by a cellular lipid transfer protein. Transfer of all tested ChOOHs was found to be markedly accelerated by SCP-2, the first-order rate constants (corrected for spontaneous transfer) decreasing in the following rank order: $7\alpha/7\beta\text{-OOH} > 5\alpha\text{-OOH} > 6\alpha\text{-OOH} > 6\beta\text{-OOH}$, *i.e.* the same as observed for spontaneous transfer (Figure 21.6).⁴⁶ In model membrane systems with SCP-2 present, the net rate constants increased dramatically with increasing ChOOH

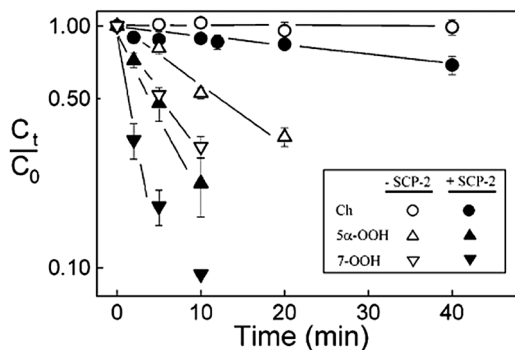


Figure 21.6. SCP-2-enhanced intermembrane transfer of ChOOHs. Reaction mixtures containing photoperoxidized RBC ghost donors and DMPC/DCP (100:1 by mol) liposome acceptors in redox-inhibited PBS either lacked or contained recombinant SCP-2. First-order kinetic plots represent analyte departure from ghosts during incubation at 37 °C. C_0 and C_t : Ch or ChOOH level at time 0 and time t . From: A. Vila, V. Levchenko, W. Korytowski and A. W. Girotti, *Biochemistry*, 2004, 43, 12592–12605, with permission.

hydrophilicity; *e.g.* the net k values for 5α-OOH and 6β-OOH were $\sim 9 \text{ h}^{-1}$ and 0.3 h^{-1} , respectively, reflecting the greater steady-state level of 5α-OOH in the aqueous compartment. SCP-2 was also shown to accelerate PLOOH transfer between membranes,⁴⁶ but the increase over background rate was substantially less than observed with 7α-OOH or 5α-OOH, for example, reflecting weaker PLOOH desorption from the donor membrane. Results of studies with cultured cells have strengthened the biological significance of LOOH translocation. For example, transfer-dependent ChOOH cytotoxicity has been demonstrated in a GPx4-null breast tumor line known to be hypersensitive to peroxidative pressure.⁴⁴ The time-dependent degree of cell killing by three different liposome-supplied ChOOHs decreased in parallel with their rates of spontaneous transfer uptake, *i.e.* 7α-OOH > 5α-OOH > 6β-OOH, thus suggesting that transfer rate-limited cytotoxicity had occurred. These cells were subsequently shown to express high levels of SCP-2, and this may have played a role in intracellular distribution of incoming ChOOHs.⁴⁴

More recent studies have provided direct support for this idea.⁴⁷ A transfectant clone (SC2A) of rat hepatoma cells stably expressing ~ 10 -times more SCP-2 than a vector control was found to be much more sensitive to liposomal 7α-OOH-induced killing. Hypersensitivity of SC2A cells was observed in both increasing [7α-OOH]/fixed time and fixed [7α-OOH]/increasing time format. Importantly, SC2A and control cells were equally sensitive to *t*-butylhydroperoxide, a nonlipid and non-SCP-2 ligand, implying that the 7α-OOH effects were SCP-2-specific. SC2A cells internalized 7α-OOH far more rapidly than vector controls and delivered more of it to mitochondria than to other compartments, suggesting preferential SCP-2-mediated delivery to mitochondria. Faster internalization and mitochondrial targeting of 7α-OOH was accompanied by greater free-radical damage, as exemplified by more

extensive (i) accumulation of 7α -OH and other one-electron reduction products, (ii) chain lipid peroxidation localized to mitochondria, (iii) loss of mitochondrial membrane potential, and (iv) induction of intrinsic apoptosis. This was the first study to demonstrate that subcellular peroxidative stress damage can be selectively targeted and exacerbated by a lipid-transfer protein.⁴⁷ Recent work has demonstrated that proteins of the steroidogenic acute regulatory (StAR) family – some of which specifically transfer Ch to/into mitochondria for steroid hormone synthesis (steroidogenic cells) or reverse Ch transport (vascular macrophages) – can also deliver 7α -OOH, once again leading to mitochondrial damage/dysfunction.^{48,49} In photodynamic systems, one can predict a similar damaging transfer scenario for 5α -OOH. This ChOOH is relatively long lived and can be generated in high yields by membrane-bound sensitizers, particularly in Ch-rich plasma membranes of target cells. These factors could favor 5α -OOH's possible toxicity-enhancing redistribution by transfer proteins such as SCP-2 and StARs. Such possibilities are not well recognized by photodynamic researchers and deserve to be carefully investigated in the interest of better understanding toxic mechanisms that underlie anticancer photodynamic therapy (PDT), for example.

21.5. Lipid Hydroperoxides as Stress-Signaling Molecules

21.5.1. Comparison of LOOH and H_2O_2 Signaling Scenarios

We have discussed the cytotoxic pro-oxidant effects of LOOHs arising from photo-oxidative stress and how these effects can be suppressed by antioxidant enzymes such as GPx4. It is now clear, however, that LOOHs may not only be cytotoxic, but at relatively low levels may act in a more subtle manner on target cells. As oxidative pressure increases, a response progression such as the following can be envisioned: (i) no net membrane damage if antioxidant capacity is not overwhelmed, (ii) relatively minor sublethal damage that may induce cytoprotective (pro-survival) responses, (iii) more extensive damage that exceeds cytoprotective capacity and triggers apoptotic or autophagic cell death, or (iv) gross membrane damage that results in necrosis because programmed apoptotic or autophagic responses are no longer possible. The tipping point between cell survival and death would occur at some point between responses (ii) and (iii). More often than not, this will involve a redox signaling cascade set off by oxidation of unique (relatively low pK_a) cysteine residues on special “sensor” proteins. Often observed is oxidation of a specific cysteine in thiolate form ($-Cys^-$) to a sulfenic acid ($-CysOH$), followed by intramolecular or intermolecular reaction with another cysteine thiol to give a disulfide, the affected protein either gaining or losing activity.⁵⁰ The key feature of this signaling event that distinguishes it from toxic oxidative modification is reversibility, *e.g.* disulfide formation can be reversed by GSH or thioredoxin. H_2O_2 is a well-known initiator of redox-signaling cascades, and much more is known about cysteine-mediated H_2O_2 signaling than

that of most other natural peroxides, including LOOHs. At low concentrations (0.1–10 μM), H_2O_2 can signal for enhanced growth in cultured mammalian cells, whereas at higher concentrations (10–100 μM), it can induce stress signaling that results in growth arrest and apoptotic death.^{51,52} Above 100 μM , H_2O_2 may produce an oxidative stress that overcomes or negates more restrained signaling activity, resulting in membrane disruption and necrosis. The average lifetime of H_2O_2 produced in cells is expected to be longer than that of free-radical precursors or products, which would favor its ability to move from generation sites (*e.g.* respiring mitochondria or NADPH oxidase on plasma membranes) to other sites for activation of sensor proteins. The signaling lifetime would depend on possible encounters with GPx1 and other neutralizing enzymes. H_2O_2 has no known cytosolic protein transporters, but is known to traverse biomembranes *via* aquaporin channels.⁵¹ It has been proposed that sensor proteins at different subcellular sites differ in their ability to recognize randomly migrating H_2O_2 and that this might at least partially explain its diverse signaling responses. However, this issue continues to be debated and remains largely unsettled.

Even less is known about LOOH-dependent signaling in this regard, but there is evidence that a pre-existing low level of LOOH, referred to as a “peroxide tone”, is necessary for cell maintenance and proliferation.^{25,45} The discovery that LOOHs like 5 α -OOH and 7 α /7 β -OOH can translocate from sites of origin has opened up new possibilities for disseminated redox signaling which differ from those applying to H_2O_2 . One key factor is that amphiphilic LOOHs should move mainly between low-polarity compartments (membrane confines) in cells, whereas hydrophilic H_2O_2 would be relegated to aqueous compartments. Accordingly, one can postulate that sensor proteins on or near membranes would be the preferred targets of mobilized LOOHs (Figure 21.7). On the other hand, relatively hydrophilic or nonmembrane-associated sensors would presumably be the preferred targets of H_2O_2 . While there are many well-defined examples in the latter category, including thioredoxins, peroxiredoxins, protein tyrosine phosphatases, and certain mitogen-activated protein kinases, membrane-associated analogs specifically activated by LOOHs are yet to be identified. As FAOOHs, PLOOHs, and ChOOHs are larger in molecular size than H_2O_2 , they would diffuse more slowly to sensor targets than H_2O_2 . LOOH transit would be even slower if mediated by transfer proteins. Beyond these considerations, there would be the following advantages in protein-mediated LOOH signaling: (i) longer LOOH lifetime due to sequestration and protection against reductive turnover in transit, and (ii) site-specific LOOH delivery dictated by preferred transfer protein–sensor protein interactions. Also, PLOOHs and ChOOHs may be more long lived than H_2O_2 because only GPx4 (or Prx6 for PLOOHs) can inactivate them *en route*, whereas H_2O_2 can be rapidly decomposed by more abundant GPx1, Prxs, or catalase. As indicated above, genuine LOOH-initiated signaling remains largely unexplored, except for two noteworthy examples: (i) a study showing that scramblase-mediated transfer of phosphatidylserine hydroperoxide (PSOOH) from the inner

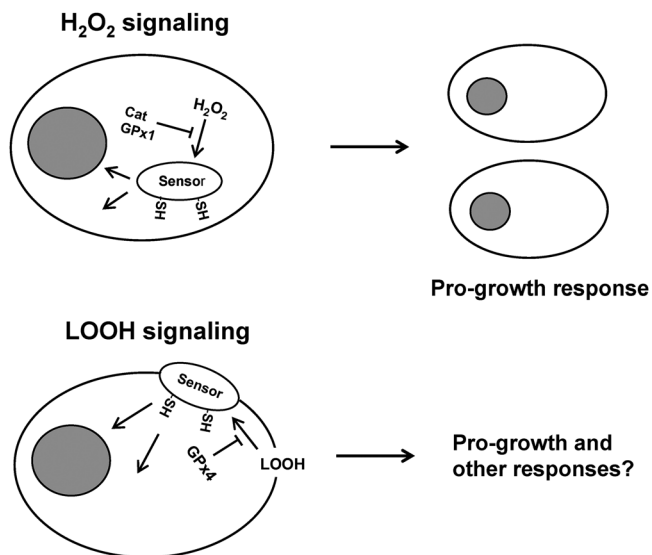


Figure 21.7. Comparison of H₂O₂ and LOOHs as intracellular redox signaling molecules. Postulated contrasting signaling mechanisms involving different protein sensors, sensor locations, and regulatory enzymes are depicted.

to outer plasma membrane leaflet of apoptosing Jurkat cells signaled for engulfment by macrophages;⁵³ and (ii) a study showing that mitochondrial oxidative stress in cardiomyocytes produced cardiolipin hydroperoxides, which translocated from the inner mitochondrial membrane to the outer, thereby serving as early apoptotic signals.⁵⁴

21.5.2. Unique Properties of 5 α -Hydroperoxycholesterol

Among the ChOOHs and PLOOHs discussed in this chapter, 5 α -OOH, a tertiary hydroperoxide (which may be biologically unique) deserves special attention because it might be the most cytotoxic, based on the following considerations. 5 α -OOH is the most rapidly generated ChOOH by ¹O₂ and the slowest to be detoxified by GPx4. As a result, 5 α -OOH should have a significantly longer average lifetime than the other ChOOHs – and also PLOOHs, which are considerably better GPx4 substrates. A relatively long lifetime would make 5 α -OOH more available to trigger chain-peroxidative damage, which could explain its unusually high cytotoxicity. A long lifetime would also favor the ability of 5 α -OOH to translocate from one membrane to another, and this could greatly expand its cytotoxic and redox-signaling range. Our understanding of these unique characteristics of 5 α -OOH is still far from complete, and much more remains to be investigated in an effort to better appreciate its role in pathologic as well as therapeutic photobiology.

21.6. Summary and Perspectives

As key intermediates of lipid peroxidation, including $^1\text{O}_2$ -induced lipid peroxidation, LOOHs are typically much longer lived than free-radical precursors or products. Once formed, LOOHs are subject to several different fates as discussed, including one-electron reduction that magnifies damage/dysfunction, two-electron reduction that attenuates it, and intermembrane translocation, after which either of these processes can ensue. If LOOH acceptor compartments are antioxidant deficient, translocation can disseminate toxic pro-oxidant effects of photo-oxidation, whereas at relatively low LOOH pressure, translocation may serve as a means of disseminating redox signaling to subcellular sites that might otherwise not experience it. Unlike H_2O_2 , LOOHs are amphiphilic and can be sequestered and shuttled to specific sites by transfer proteins, suggesting that their underlying signaling mechanisms may be quite distinct from those of H_2O_2 , which diffuses randomly and has no known transporters. Spontaneous and protein-mediated LOOH transfer might also play a role in stress-induced bystander effects, both within and between cells. As in the case of H_2O_2 , LOOHs are no longer regarded solely as cytotoxic species, but rather as relatively long-lived secondary products of oxidative (including photo-oxidative) stress with a potentially rich array of signaling activities that could complement those of H_2O_2 . Most of these activities remain to be elucidated and pursuing this will be truly exciting.

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Chapter 22

Reactions of Singlet Oxygen with Organic Devices

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22.1. Introduction

An organic device can be generally described as an assembly of compounds that fulfil functions in optical and electronic applications.¹ Such devices can operate as thin film transistors,² solar cells,³ light-emitting diodes (LEDs),⁴ sensors⁵ and photo switches.⁶ These functions rely on an extended conjugated π -system built up as a conjugated polymer or as an aromatic molecule.^{7,8} The reaction of the π -system with $^1\text{O}_2$ is deemed to be harmful as any oxidation would deteriorate the operating performance of the device.⁹ However, some applications deliberately involve $^1\text{O}_2$ reactions.¹⁰ Owing to the spatially addressable generation of the reactive species by irradiation (*e.g.* by a laser beam or through a mask), patterning techniques were developed where the device material is destroyed for demarcation. A further incentive is provided by the reversibility of the [4+2] cycloaddition of $^1\text{O}_2$ to acenes.¹¹ This allowed writing and erasing data by reversibly adding dioxygen to a device material.

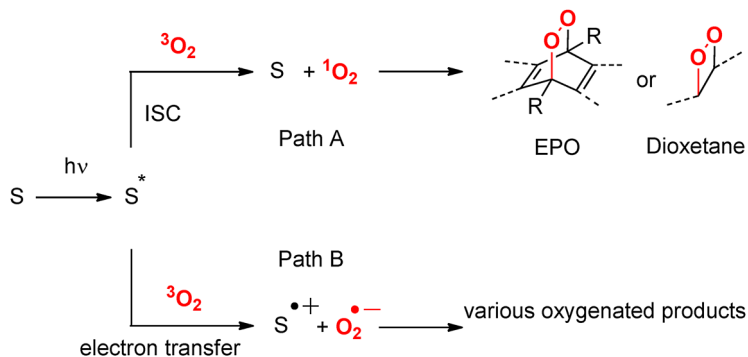
This chapter will address both, the destructive and the beneficial character of $^1\text{O}_2$ in terms of device applications. We will begin in Section 22.2 with a description of the degradative processes in organic devices, including the most important affected materials. At the end, methods are presented for the stability enhancement. In Section 22.3 lithographic techniques involving $^1\text{O}_2$ are presented, which can be divided into destructive and nondestructive methods. Section 22.4 deals with photochromic compounds and photoswitches.

22.2. Degradative Reactions of Singlet Oxygen within Organic Devices

Most studies of oxidative degradation of electronic and optical organic devices have been conducted with polymers, such as poly(1,4-phenylenevinylene) (PPV), poly(3-alkylthiophenes) (P3AT) and small aromatic molecules, namely, polyaromatic hydrocarbons (PAH). Photo-oxidations of such materials are accompanied with a shift and decrease of the absorption and emission wavelength as their π -conjugation becomes disrupted. In turn, the device performance deteriorates. This sensitivity is not only restricted to solutions but also occurs in the solid state if sufficient diffusion of oxygen is permitted.

22.2.1. Possible Mechanistic Pathways of Photo-Oxygenations

There are two competing mechanisms describing the oxidative photodegradation: in the first, the light-exposed material sensitizes the generation of singlet oxygen (see Scheme 22.1, path A).¹² This requires that the triplet energy of the material is higher than 0.98 eV, which equals the energy barrier from $^3\Sigma_g$ to $^1\Delta_g$ O_2 . The reactions are encompassing basically [2+2] or [4+2] cycloadditions between $^1\text{O}_2$ and the π -systems giving dioxetanes or



Scheme 22.1. Possible pathways of photodegradation reactions.

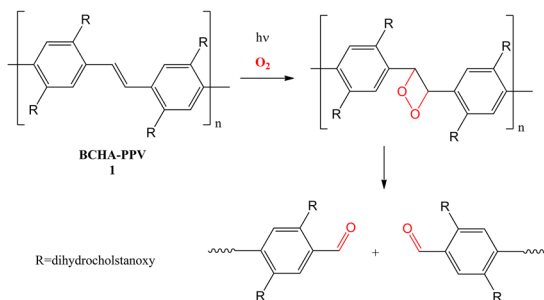
endoperoxides (EPOs), respectively. Ene reactions for such systems are less likely owing to the lacking of allylic hydrogen atoms.¹³ In the second mechanism, an electron transfer (ET) from the excited species to O_2 generates a radical cation and superoxide (see Scheme 22.1, path B).¹⁴ The rate of this ET would correlate with the LUMO energy of the species and the reaction would commence with a C–O bond formation according to a stepwise radical process.¹⁵ Furthermore, it has to be noted that also other photodegradation processes are taking place that do not involve oxygen, such as dimerization reactions.¹⁶ Those reactions are usually proceeding at a slower rate and become considerable only at deficiency of oxygen.

22.2.2. Photo-Oxidative Degradation of PPVs

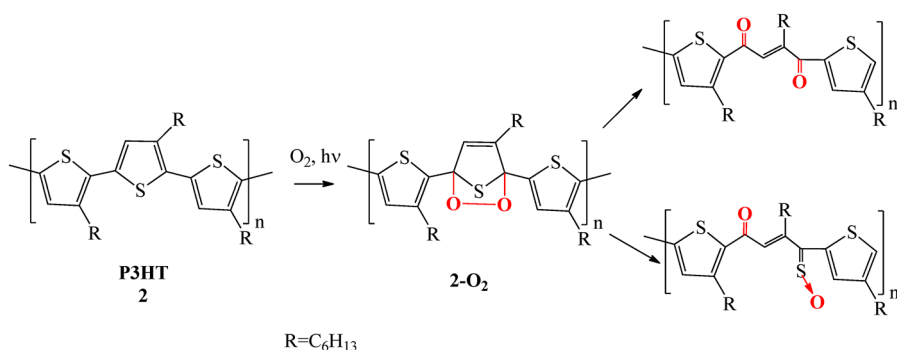
The degradation mechanism of photoluminescent PPVs was investigated on the well-soluble poly(2,5-bis(5,6-dihydrocholestanoxyl)-1,4-phenylvinylene) (BCHA-PPV) (**1**).¹⁷ Singlet oxygen was detected upon illumination in solution arising from an energy transfer of the BCHA-PPV triplet state. It was proposed that $^1O_2$ adds in a [2+2] cycloaddition to the double bond to give dioxetane groups (see Scheme 22.2). Macromolecular chain scission is ensuing from the ring cleavage. The mechanism is consistent with the observed decrease of olefinic C–H absorption and increase of carbonyl absorption in FTIR spectra.¹⁸

22.2.3. Photo-Oxidative Degradation of P3ATs

It was reported that excited triplet states are generated by ISC of singlet excitons upon illumination of several P3ATs that in turn generate $^1O_2$ by sensitization.¹⁹ Others proposed that $^1O_2$ is generated upon dissociation of an excited thiophene– O_2 CT complex.²⁰ Hence, the thiophene moieties in P3HT (**2**) are oxidized to transient EPOs (**2–O₂**) that are known to give sulfines or diketones in solution (see Scheme 22.3).²¹



Scheme 22.2. Suggested mechanism of the photo-oxygenation and cleavage of BCHA-PPV.



Scheme 22.3. Mechanism of the photo-oxygenation of P3ATs.

Relatively few studies were undertaken to characterize the decomposition products in illuminated P3ATs. From the appearance of C=O stretching vibrations in IR spectra of the solid material it was inferred that carbonyl groups are formed.^{20,22} Based on the identified structures, the thioanhydride **3** and the substituted terthiophene **4**, obtained from isolated products of an irradiated P3AT film, an additional reaction path involving biradical **5** was suggested (see Figure 22.1).²³

22.2.4. Photo-Oxidative Degradation of PAHs

Theoretical calculations predict that larger PAH exhibit an increasing open-shell diradical character that is reflected in an increase of the total spin.²⁴ Indeed, PAH larger than pentacene are oxidized with $^3\text{O}_2$ in the dark.²⁵ The mechanistic course of the [4+2] cycloaddition of $^1\text{O}_2$ to PAH is therefore undergoing a transition from a concerted to a stepwise process along the series from benzene to large PAHs.

The reaction between pentacene (**6**) and singlet oxygen in solution gives exclusively the 6,13-EPO **6-O₂**, which further undergoes thermal

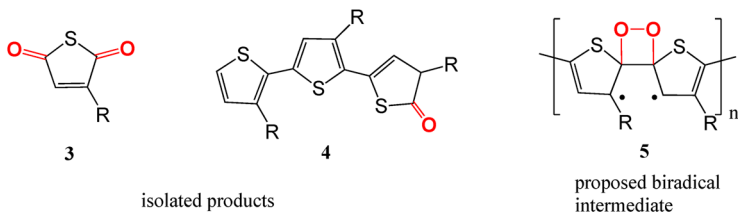
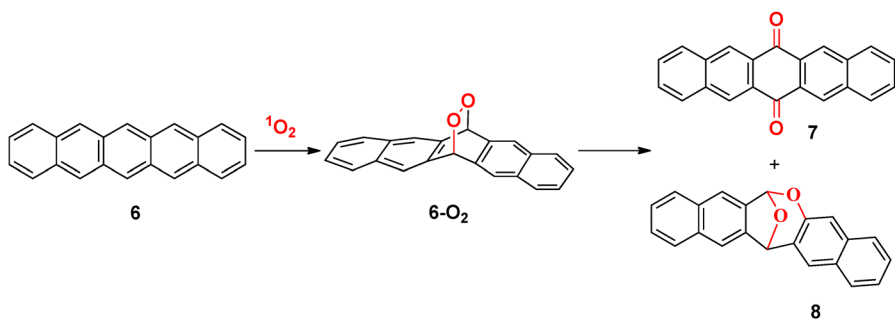


Figure 22.1. Structures of the decomposition products of P3ATs.



Scheme 22.4. Products resulting from the photo-oxygenation of pentacene (6).

decomposition to give 6,13-pentacenequinone (7) and the bicyclic acetal 8 (see Scheme 22.4).²⁶

Thin solid films of pentacenes are unexpectedly persistent to oxidative degradation as compared to substituted pentacenes and tetracenes that degrade faster as pentacene in the solid state but more slowly in solution.^{27,28} Grazing-incidence X-ray diffraction (GIXD) and atomic force microscopy (AFM) investigations of pentacene monolayers on dielectric substrates unveiled that the oxidation process is confined to edges at boundaries that grow during long-time aging.²⁹ The products formed after a pentacene film was exposed to light, oxygen and air were analyzed by LDI-TOS mass spectroscopy and revealed mass increases of 15, 16, 30, 31, 32 and 46.³⁰ Mass increases of +32 and +30 correspond to EPO 6-O₂ and quinone 7, respectively, supporting an ¹O₂ mechanism. However, mono- and trioxxygenated products should originate from other pathways.

22.2.5. Stability Enhancement of Optical Devices

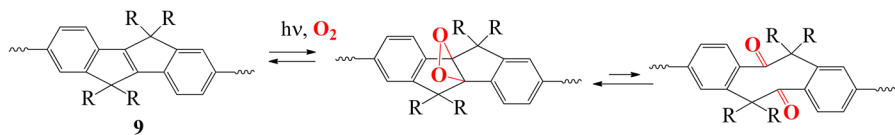
There are several options to protect PAH and polyolefins from oxidation that include imposing rigidity and endowing with certain stabilizing functional groups. In most cases, however, the modification causes a blue shift of the absorption and emission spectra as the optical and electrical performances are reduced.

Stabilization by rigidity was accomplished with the monomeric unit **9** being incorporated as a comonomer in PPVs used for the fabrication of solar cells. Cyclization of the vinylene groups to a five-membered ring yielded in a strong improvement of the copolymer to endure illumination under air. The increased stability against photo-oxygenation was explained by the reversibility of dioxetane cleavage to the diketone (see Scheme 22.5).³¹

The electron-withdrawing CF_3 group can be partially attached to the benzene rings in PPVs to give the photo-oxidatively more stable copolymerized PPVs **10** (see Figure 22.2).^{32,33} The number of CF_3 substituted comonomers is limited by the solubility.

If PAHs are endowed with alkynyl substituents a significant stabilization towards the [4+2] cycloaddition with singlet oxygen can be achieved.²⁸ The effect can be impressively demonstrated by comparison between alkynyl- and unsubstituted PAHs of different ring sizes (see Figure 22.3).³⁴

The 6,13-(triisopropylsilyl)ethynyl)pentacene (**11**) is suitable for solution-processed OTFT fabrication with field-effect mobilities $>1 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$.³⁵ The origin of the stabilization effect and the operative mechanism were intensively discussed on the basis of a low-lying LUMO energy that makes the ET from the excited LUMO less likely and a low triplet energy that precludes a singlet oxygen sensitization.^{28,36} It was finally proven in our group on the basis of a “dark oxidation” using a chemical source of $^1\text{O}_2$, that **11** reacts indeed with $^1\text{O}_2$ to give a 6,13-endoperoxide (**11-O₂**) with a rate constant 4000 times smaller than that of pentacene (**6**) (see Figure 22.3).³⁴ From competition experiments it also became clear that $^1\text{O}_2$ is physically deactivated by **11** to an enormous extent. The slow reaction of **11** is best explained by the reversible formation of an exciplex that collapses only slowly to the EPO **11-O₂** (see Scheme 22.6).³⁴ Under irradiation, an ET mechanism gains importance explaining why the reactivity of differently substituted alkynyl-pentacenes is controlled by the LUMO energy.^{15,28}



Scheme 22.5. Deployment of fused PPVs to evade cleavage after photo-oxygenation.

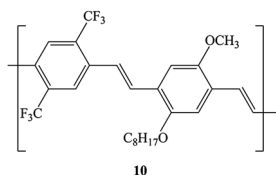


Figure 22.2. Structure of PPVs carrying CF_3 stabilizing groups.

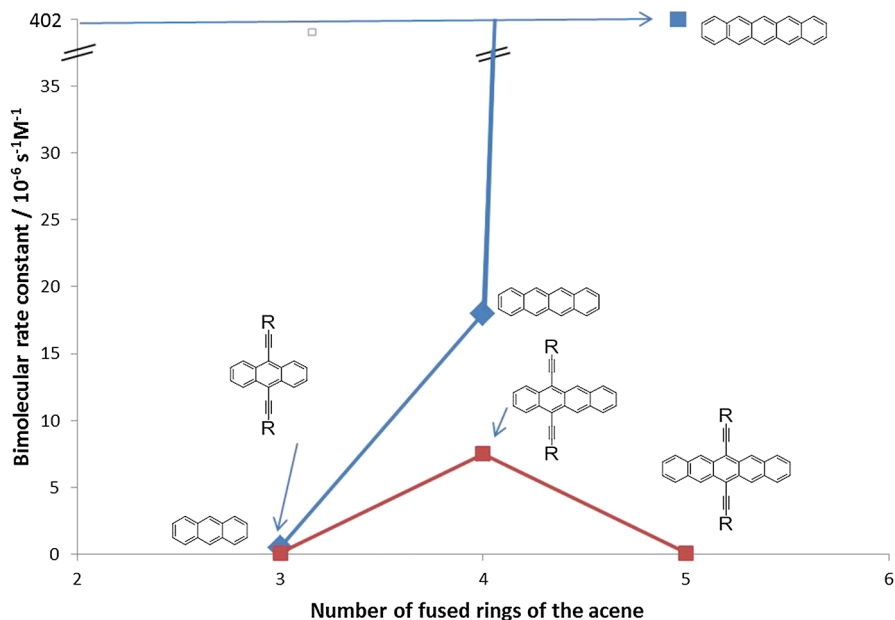
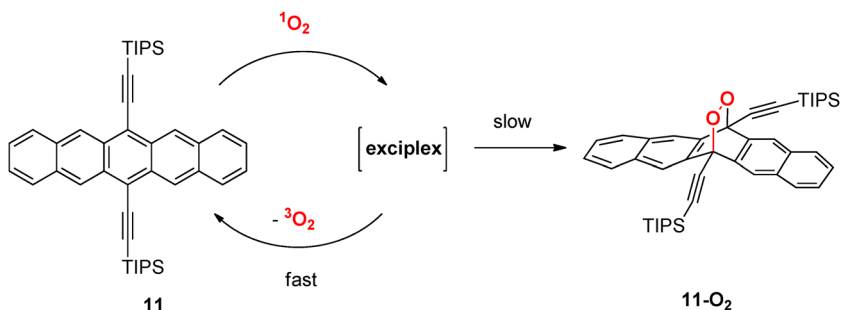


Figure 22.3. Substituent and ring-size effects on the rate of photo-oxygenations of PAHs. With permission of the American Chemical Society 2012.



Scheme 22.6. A “slow” photo-oxygenation of alkyne pentacene **11** via a reversible exciplex formation.

The strongest stabilization towards photo-oxidation of PAH is harnessed from sulfur groups.³⁶ Alkylthio- and arylthio-pentacenes **12** and **13** (see Figure 22.4) persist under the same conditions longer to exposure than alkyne pentacene **11**.

Since the sulfur and alkyne group have similar impacts on the energies of HOMO and LUMO it was suggested that the sulfur group could physically deactivate $^1\text{O}_2$ even more than the alkyne group.³⁶ On the other hand, placement of thio groups impacts the total spin $\langle S^2 \rangle$ of the PAH as a high total spin becomes more prone to follow a stepwise reaction path with either $^1\text{O}_2$ or $^3\text{O}_2$.³⁷

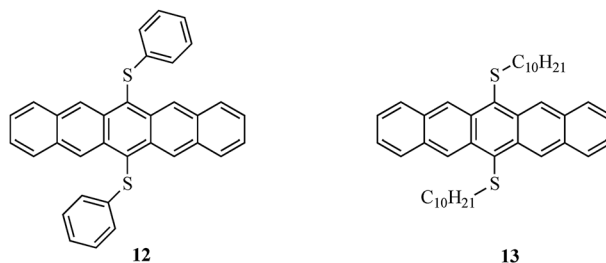


Figure 22.4. Structures of PAHs stabilized with sulfur groups.

22.3. Lithography with Singlet Oxygen on Organic Materials

Singlet oxygen lithography describes procedures where $^1\text{O}_2$ is generated within a deposited film by irradiation under air to react with the material to a more (positive tone) or less (negative tone) soluble form that can be developed to give an image. The technique can be subdivided into two categories: irreversible (destructive) photopatterning where the exposed material is lost owing to decomposition or crosslinking and regenerative photopatterning with recovery of the exposed original material by means of reversible $^1\text{O}_2$ reactions. We will begin with examples that belong to the classes of genuine photoresists rather than functional devices materials for optical or electronic applications.

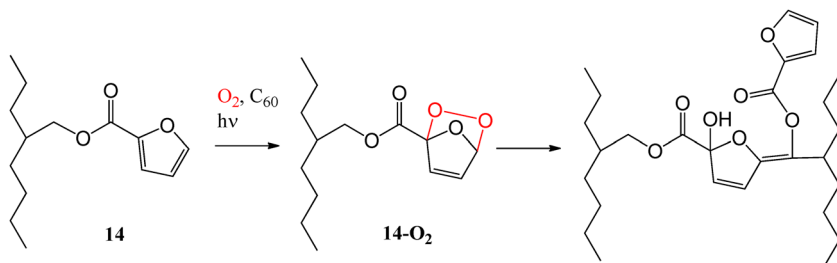
22.3.1. Irreversible Photolithography with $^1\text{O}_2$

Breslow developed a negative-tone photoresist composed of a sensitizer, an allylic olefin and a monomer that was suitable for the application as photoresist.³⁸ Upon irradiation, the allylic olefin underwent an ene reaction to form a hydroperoxide that functioned as initiator of a radical polymerization. Thus, $^1\text{O}_2$ delivered the insoluble part of the pattern.

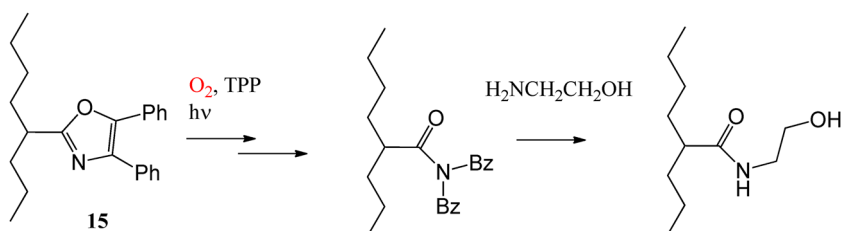
Various polymers endowed with furan rings (**14**) at the end of their side chains were used as negative-tone photoresists using fullerene as sensitizers. It was asserted that a furan endoperoxide is formed in a [4+2] cycloaddition that undergoes thermal condensation by dehydration to give an insoluble crosslinked polymer (see Scheme 22.7).^{39,40}

In contrast, a positive-tone photoresist was designed with a terminal 1,3-oxazole group at the side chains (**15**).^{41,42} Photo-oxidation leads *via* a transient endoperoxide to a carboxamide. The dissolution of the exposed material was achieved after dipping into an aqueous 2-aminoethanol solution (see Scheme 22.8).

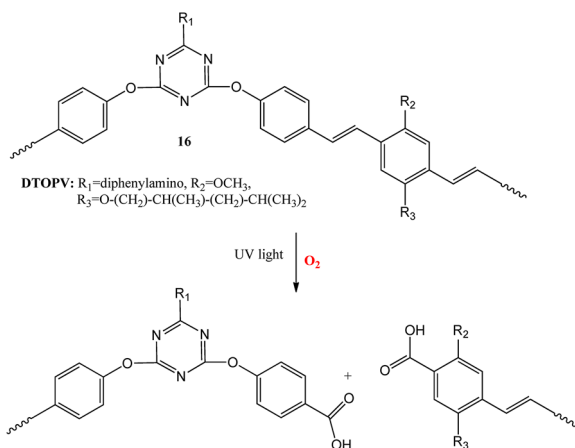
The fluorescent *S*-triazine bridged *p*-phenylenevinylene polymer (DTOPV) (**16**) was employed to generate pattern structures upon UV illumination under air.⁴³ A strong increase of the hydrophilicity of the exposed areas was detected that was explained by the formation of negatively charged



Scheme 22.7. Induced photocrosslinking of furan film by $^1\text{O}_2$.



Scheme 22.8. Principle of a $^1\text{O}_2$ -based positive-tone lithography using oxazole groups.



Scheme 22.9. Mechanism underlying the $^1\text{O}_2$ -based photopatterning of DTOPV.

carboxylic acid groups (see Scheme 22.9). Those formed after a [2+2] cycloaddition similar to give a dioxetane (see Scheme 22.2 in Section 22.2.2). It was demonstrated that mesenchymal stem cells (MSC) could be attached at the irradiated regions that showed a proliferation rate being higher than the rate on the unexposed regions (see Figure 22.5).

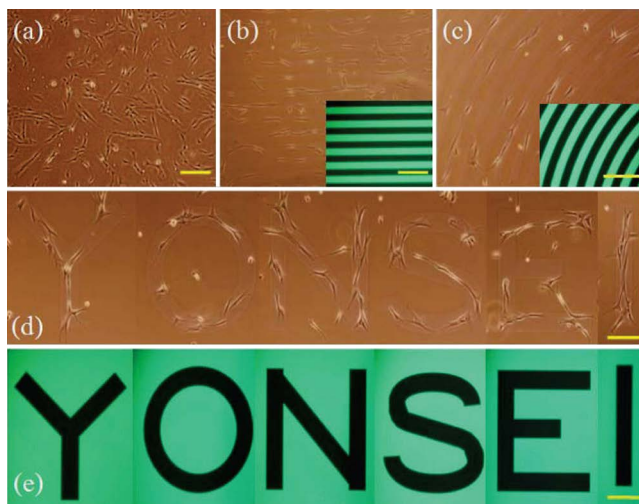


Figure 22.5. Optical microscope images of MSCs cultured on (a) tissue-culture polystyrene, (b) 50 μm wide line pattern of DTOPV film, (c) 50 μm wide curved line pattern of DTOPV film, (d) 100 μm wide “YONSEI” letter pattern with MSCs on DTOPV film, and (e) fluorescent microscope image of the same “pattern with cells”. Fluorescent pattern images are inserted in parts (b) and (c). Scale bar = 200 μm . With permission of the American Chemical Society, 2009.

22.3.2. Regenerative Photolithography with $^1\text{O}_2$

Reversible reactions between singlet oxygen and PAHs found successful applications in lithography. The alkyloxyphenylanthracene **17** forms glassy transparent films as crystallization is retarded owing to the long alkyl chain.⁴⁴ Upon irradiation under air through a photomask anthracene **17** reacts in a [4+2] cycloaddition with $^1\text{O}_2$ to give the EPO **17-O₂**. Following a developing step using hexane to etch off the less-polar parent anthracene, a free-standing pattern motif of **17-O₂** is created (see Figure 22.6). After a subsequent heating step the parent anthracene can be recovered.⁴⁴ A film of **17-O₂** deposited on silver appeared sufficiently stable to protect the underlying silver layer from etching with HNO_3 .⁴⁵

22.4. Reactions of Photochromic Devices and Switches with Singlet Oxygen

Photochromism describes the reversible change of the color of a molecule upon action of light or heat.^{6,46} PAHs that undergo a reversible [4+2] cycloaddition with $^1\text{O}_2$ to colorless nonfluorescent EPOs (see Scheme 22.10) are complying with this description.¹¹ An eminent example is 9,10-diphenylanthracene (DPA) (**18**) that reacts rapidly to the EPO **18-O₂** upon irradiation by

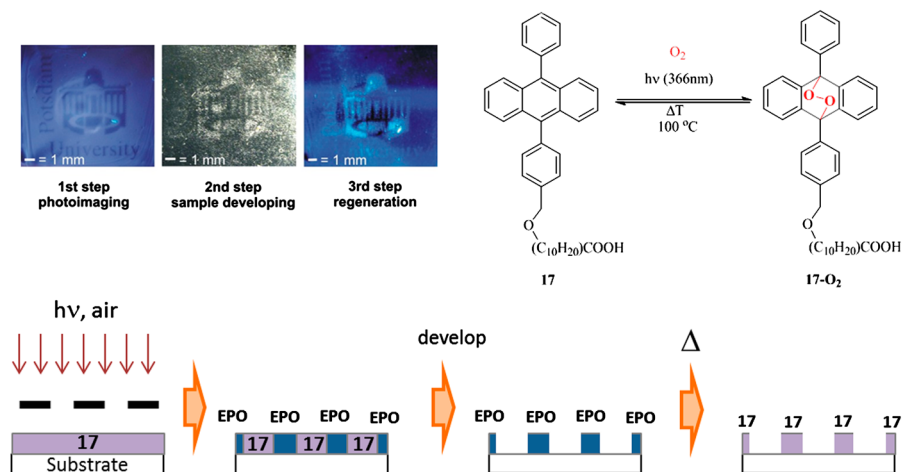
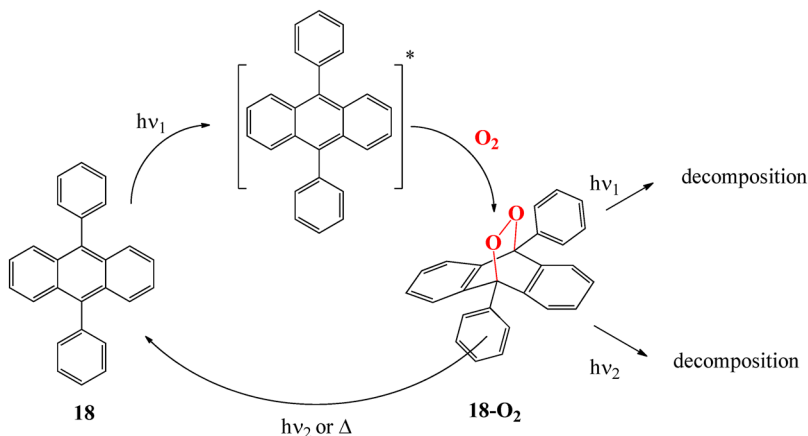


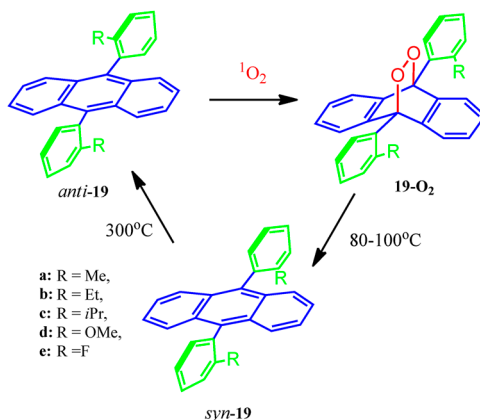
Figure 22.6. Application of anthracene **17** as photoresist that is recovered in the final step.



Scheme 22.10. Action of DPA as photochromic compound.

¹O₂ self-sensitization. The EPO reconverts quantitatively at >100 °C to the initial form.¹¹ The phenyl substituents at the centered (9,10) carbon atoms play a crucial role during the cycloreversion to favor the C–O cleavage over the destructive C–C cleavage. Hence, PAHs with hydrogen, oxygen or alkyl substituents attached at these carbon atoms react irreversibly to EPOs that decompose upon heating.³⁴

The same reaction principle was applied to design a molecular rotary switch, where the anthracene unit served as stator, while the adjacent benzene rings acted as the rotating units.^{47,48} The substituents at the *ortho* position of diarylanthracenes **19** can be either located at opposite sites with



Scheme 22.11. Application of anthracene **19** as a molecular switch flipping between *anti* and *syn* conformation by reaction with ¹O₂ via the EPO **19-O₂**.

respect to the anthracene plane (*anti*-conformation) or at the same site (*syn*-conformation). These two stages are rotationally stable in the dark up to 200 °C but interconvert from *anti* to *cis* upon irradiation under air (see Scheme 22.11). At the first stage, the EPO **19-O₂** is formed from *anti*-**19** via a 180° rotation along the aryl–aryl axis. In the second stage *syn*-**19** is formed by thermal reconversion of the EPO. Finally, *anti*-**19** is retrieved by thermal interconversion at 300 °C.

Use of the alkyloxyphenylanthracene **17** (see Section 22.3.2) allowed to produce thin fluorescent films on glass that was harnessed to write by irradiation, erase by heating and re-write by irradiation (see Figure 22.7).⁴⁹

For all EPOs of PAH the reconversion by irradiation entails a loss of material that is caused by inadvertent cleavage of the O–O bond.⁵⁰ This limits their application as photochromic materials solely operating by irradiation. One exception can be found with heterocoerdianthrones (HCD) that have proven to suit as genuine photochromic materials since decompositions are fully averted due to their structural rigidity.⁵¹ The EPO of HCD **20** was used as a reusable liquid actinometer in the UV region of 248–334 nm with a straightforward calculability of the radiation quantum flux (see Scheme 22.12).⁵²

22.5. Conclusions

In this chapter, we presented the advantageous and deleterious aspects of singlet oxygen reactions in organic functional devices. Such device materials are synthesized from conjugated polymers and PAHs. The generic course of material degradation is commencing with a photoexcitation of the material followed by energy transfer to ground-state oxygen. Singlet oxygen is then added in either [2+2] or [4+2] cycloadditions to the π -systems. The oxidation stability can be enhanced by introduction of substituents such as CF₃, alkynyl or thio

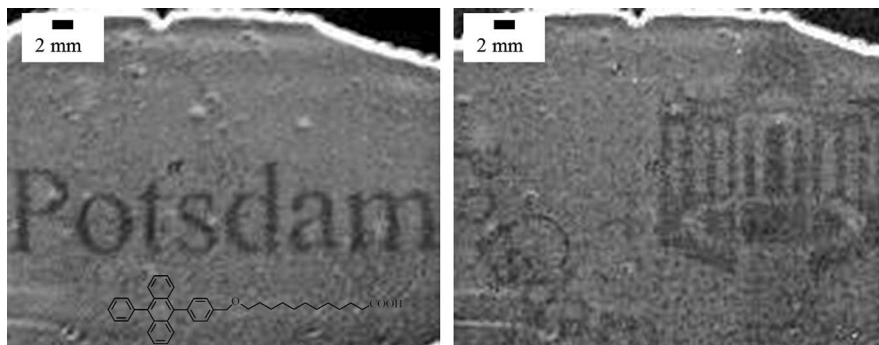
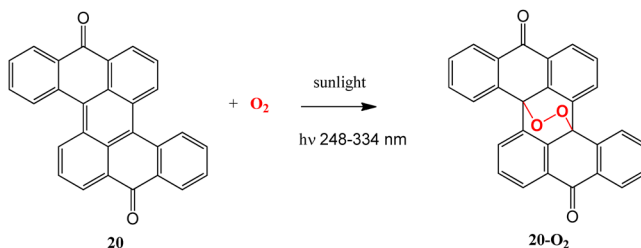


Figure 22.7. Fluorescence images showing writing, erasing and rewriting on a thin film of alkyloxyphenylanthracene **17**. With permission of the American Chemical Society, 2005.



Scheme 22.12. Photo-oxygenation of HCD **19** to the EPO **19-O₂**.

groups. Some lithographic applications rely on photo-oxygenations rendering the exposed material either more or less soluble. A particular advantage is brought by the reversible [4+2] cycloaddition of $^1\text{O}_2$ to PAHs. A sustainable lithography can be carried out without decomposition of the material and the reversibility of the reaction allows its application for data storage.

The beneficial applications are in a pioneering stage since they are still restricted on the smallest members of PAH, furans and oxazoles, which cannot be considered as fully fledged functional materials. It is therefore a pending challenge to design reversibly reacting device materials with high performance.

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Chapter 23

Singlet Oxygen Mediated Photodegradation of Water Contaminants

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23.1. Sensitized Photodegradation as a Tool for Natural Waters' Depuration

Nowadays, the quality of natural waters is a matter of great concern. As a result of human activities many organic compounds are released to rivers, lakes and seas, a fact that constitutes a serious risk due to the possible adverse effects to living organisms. In this context, a typical case is given by the high amounts of agricultural pesticides, contaminants of surface waters and soils, constituting a serious potential risk for groundwater resources.

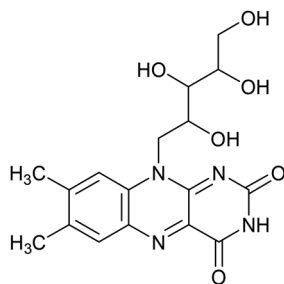
Self-depuration processes that can help to handle with this issue are thermal and photochemical degradation. Specifically, photopromoted degradation under natural conditions looks, in theory, like an ideal setting. This environmentally friendly combination has been tested, under field and laboratory conditions, with varying success. As a result, a high variety of the reactions involved have been evaluated in order to know more about the photochemical evolution of water pollutants (for reviews on the topic see ref. 1–4).

Photoinduced degradation constitutes a decay process that depends on the energy supplied by light irradiation to generate electronically excited species and the subsequent breakage of molecular bonds to form a new substance. The study of organic contaminants photodegradation in aquatic ecosystems has dealt with the inconvenient that most of those pollutants are colorless and cannot be degraded by sunlight illumination. In the last decades, great efforts were focused on getting more insight in the mechanisms of decomposition of those compounds that are transparent or quasitransparent to daylight.

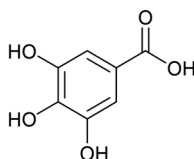
The contact between natural waters and soils generates a heterogeneous mixture of daylight-absorbing organic compounds – including the so-called *native photosensitizers* – which can play an important role in pollutants decomposition under environmental conditions. In aerated media, and in general terms, the mechanism of photodegradation occurs upon absorption of visible light by a sensitizer that can, in an initial step, react with dissolved oxygen-generating reactive oxygen species (ROS), such as singlet molecular oxygen $^1\text{O}_2$, superoxide radical anion ($\text{O}_2^{\cdot-}$), hydroxyl radical ($\text{HO}^\cdot$) and hydrogen peroxide (H_2O_2) among others. Hopefully, this step is followed by an oxidative decomposition of the contaminant.^{5,6} In parallel, the excited sensitizer can react straightforward with the target molecule, generally through an electron-transfer mechanism.^{6,7}

It is well known that the presence in nature of colored mixtures of organic compounds such as humic substances (HS), including humic acid (HA) as one of the main components, and/or traces of riboflavin (Rf, vitamin B₂; chemical structure in Scheme 23.1) that can absorb solar radiation and subsequently act as a photosensitizers.^{8–12}

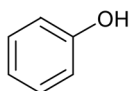
The naturally occurring HS constitute the predominant fraction of such organic compounds. They are formed by microbial degradation of dead plants and are broadly distributed in terrestrial and aquatic ecosystems.^{13,14}



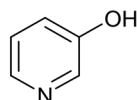
Riboflavin (Rf)



Gallic Acid (GA)



Phenol (Ph)



3-hydroxypyridine (3-OHP)

Scheme 23.1. Chemical structures of the most relevant molecules described in the chapter.

Rf constitutes a daylight-absorbing pigment of particular interest mainly due to its presence as traces in water courses, lakes and seas. It is synthesized by green plants and participates in a variety of enzyme-catalyzed oxidation-reduction reactions.¹⁵ The vitamin has been repeatedly postulated as a sensitizer for the natural photo-oxidative degradation of water contaminants, in particular those resulting from agricultural activities.^{4,16}

In the photochemical transformation of organic contaminants, the molecules of native sensitizers such as the mentioned Rf and/or HS initially absorb energy from sunlight to populate electronically excited states that, in turn, generate ROS.

23.2. On the Environmental Relevance of ¹O₂ in the Context of Water Contamination

Several research studies propose that among the ROS generated in aquatic systems, ¹O₂ has considerable importance, due to its steady-state concentration and lifetime.^{4,9,10,17–20} In the environment, the oxidative species is almost exclusively produced by photochemical pathways. In fact, Rf possess a quantum yield for ¹O₂ generation of 0.47 in aqueous media.²⁰ Besides, as relatively high concentrations of ¹O₂ were found in intensely colored natural waters, it can be deduced that one of the active sensitizing compounds in aquatic ecosystems might be HS, leading to estimated steady-state concentrations of the oxygenated species in the order of 10^{–12}–10^{–14} M.^{21–23} Artificially prepared humic solutions optically matched to an equivalent

absorbance at 366 nm with natural waters, showed similar quantum yields of $^1\text{O}_2$ generation employing sunlight illumination. These facts reveal the environmental significance of naturally occurring compounds in the formation of this oxidative species. Several literature reports agree that roughly 1% of the daylight absorbed by all kinds of dissolved organic compounds produces $^1\text{O}_2$.^{23,24}

23.3. Riboflavin-Sensitized Photo-Oxidation of Gallic Acid and the Possible Role of $^1\text{O}_2$ in the Generation of Humic Substances

The reactions involved in natural synthesis of HS are still not completely elucidated. Nevertheless, some evidence exists in the sense that among the main processes that can take place, the oxidative polymerization of polyphenols could be implicated.^{25,26} Within the frame of possible photoreactions, precursory in the generation of HS, the interaction between Rf and gallic acid (GA; chemical structure in Scheme 23.1) has been studied under visible-light irradiation.²⁷ GA is a colorless water soluble phenolic acid, formed in nature after lignin degradation.²⁸ A kinetic and mechanistic study concludes that GA suffers a fast photo-oxidation in pH 7 aqueous solutions.²⁷ Figure 23.1 shows the relative rates of oxygen photoconsumption

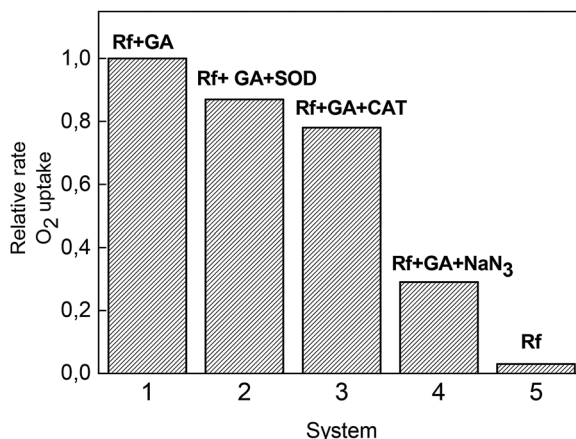
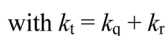
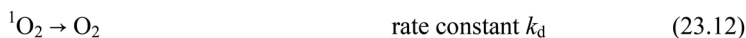
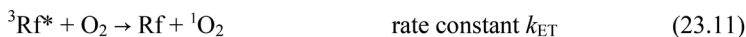


Figure 23.1. Relative rates of oxygen uptake upon visible-light irradiation by the systems: 0.02 mM Rf plus 5 mM GA (1); 0.02 mM Rf plus 5 mM GA plus $1\ \mu\text{g ml}^{-1}$ SOD (2); 0.02 mM Rf plus 5 mM GA plus $1\ \mu\text{g ml}^{-1}$ CAT (3); 0.02 mM Rf plus 5 mM GA plus 2 mM NaN_3 (4); 0.02 mM Rf (5) in pH 7 aqueous solution. Data reprinted from A. Pajares, M. Bregliani, M. P. Montaña, S. Criado, W. Massad, J. Gianotti, I. Gutiérrez and N. A. García, Visible-light promoted photoprocesses on aqueous gallic acid in the presence of riboflavin. Kinetics and mechanism, *J. Photochem. Photobiol., A*, 2010, **209** (2–3), 89–94, Copyright 2010, with permission from Elsevier.

by the system Rf + GA upon visible-light irradiation, in the absence and in the presence of NaN₃, superoxide dismutase (SOD) and catalase (CAT), all specific ROS interceptors of ¹O₂, O₂^{•-} and H₂O₂, respectively. Results demonstrate the participation of the mentioned ROS in the photoprocess.²⁹⁻³¹ It is well known that these ROS are generated by electronically triplet excited Rf after energy-transfer and electron-transfer processes.³¹ This well-known mechanistic behavior is represented by the self-explanatory set of reactions 23.1–23.14 (Scheme 23.2), which includes the possible steps in the Rf-sensitized photo-oxidation of a hypothetical electron donor species Q, where Q represents GA.^{6,7}

The k_r/k_t (Scheme 23.2) ratio may be assumed as a measure for the actual photodegradability of GA. In the present case, both the absolute values for the rate constants k_t and k_r , and the relatively high k_r/k_t ratio indicate the importance of the ¹O₂-mediated process. The general conclusion is that in natural waters, GA can undergo spontaneous and effective photodegradation under visible-light irradiation. Humic products could be produced through condensation/polymerization reactions, with participation of radical species and ¹O₂, all photogenerated in the presence of Rf.



Scheme 23.2. Possible reaction steps in the Rf-sensitized photo-oxidation of a hypothetical electron-donor species Q.

23.4. Humic Substances and Riboflavin as $^1\text{O}_2$ Sensitizers in Water-Contaminant Photodegradation

As said in Section 23.1, the colored HAs and Rf play a relevant role in the photogeneration of ROS in surface waters.^{17,18,20} This simultaneous presence of several natural light absorbers complicates the prediction of mechanism and efficiency of the eventual degradative photoprocesses involved. Indeed the problem itself constitutes a no simple task. In order to deal with this difficulty, efforts were directed to elucidate the photosensitizing effect given by the mixture of HAs and Rf.^{18,21} Recently, a systematic study on the photodegradation effect exerted by the mentioned mixture on hydroxyaromatic substrates (OHArS) was carried out.¹⁸ The compounds phenol (Ph) and 3-hydroxypyridine (3-OHP) were used as model pollutants for surface waters contaminated with compounds profusely employed in the formulation of pesticides and constituents of petroleum residues.^{4,32,33} Relevant results of this study will be briefly overviewed in order to understand, as far as possible, mechanistic aspects of the photodegradation processes involved, and the potential utility of the results obtained. The chemical structures of Ph and 3-OHP are shown in Scheme 23.1. These OHArS scarcely absorb in the spectral region of sunlight irradiation. As a consequence, any natural photopromoted process requires the presence of a sensitizer. On the other hand, too high concentrations of colored substances may act as inner filters for daylight (Figure 23.2, inset A), impeding phototransformations. In order to prevent these possibilities, low concentrations of HA + Rf were used for photodegradation studies. In such a way, the mixture HA + Rf, employed as a daylight photosensitizer, constitutes an acceptable representation of superficial layers in natural waters, where the dissolved impurity level is not sufficiently high to attain optically dense conditions.

The visible-light irradiation of solutions of the OHArS in the sub-mM concentration range, in the presence of HA and/or Rf, evaluated through UV-vis spectroscopy and oxygen photoconsumption brought the following results:

- In pH 7 aqueous solution, 3-OHP is photodegraded, whereas Ph remains unchanged. When the photosensitizing mixture was replaced by HA, the same result was observed. Nevertheless, in the same experiment carried out in pH 11 aqueous medium, both contaminants, now in their respective ionized forms, were degraded. The photodegradation of the pyridinic compound is totally suppressed by the presence of NaN_3 , a well-known physical quencher of $^1\text{O}_2$.³⁴ Parts of these results are illustrated in Figures 23.2 and 23.3.
- The rate of oxygen uptake by 3-OHP, employing the sensitizing mixture Rf + HA was neatly decreased by the presence of NaN_3 and SOD. When only HA acted as a sensitizer of 3-OHP degradation, the rate of oxygen consumption was only decreased by the presence of NaN_3 . No inhibition was observed in the individual presence of SOD, CAT and mannitol, the latter a well-known scavenger of the species hydroxyl radical.³⁵ Parts of these results are shown in Figure 23.3.

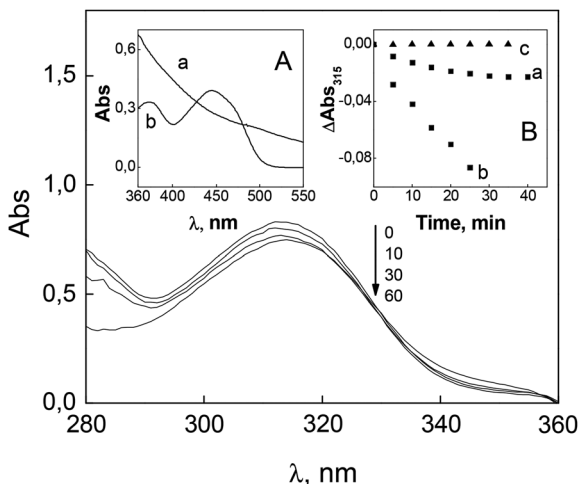


Figure 23.2. Spectral changes in a pH 7 aqueous solution of 0.2 mM 3-hydroxypyridine upon photoirradiation in the presence of 0.04 mM Rf plus $50 \mu\text{g ml}^{-1}$ HA. Numbers on the spectra represent irradiation time, in min. Inset A: absorption spectra of pH 7 aqueous solutions containing $50 \mu\text{g ml}^{-1}$ HA (a) and 0.04 mM Rf (b). Inset B: absorbance changes at 315 nm as a function of photoirradiation time of a pH 7 aqueous solution containing 0.03 mM 3-OHP plus 0.5 mM HA (a). The same as (a) for a pH 11 aqueous solution (b). The same as (b) in the presence of 5 mM NaN_3 (c). Reprinted from A. Pajares, M. Bregliani, J. Natera, S. Criado, S. Miskoski, J. P. Escalada and N. A. García, Mechanism of the photosensitizing action of a mixture humic acid–riboflavin in the degradation of water contaminants, *J. Photochem. Photobiol., A*, 2011, **219** (1), 84–89, Copyright 2011, with permission of Elsevier.

These experimental findings and earlier reports from ourselves demonstrate that the species $\text{O}_2^{\cdot-}$ is generated through processes 23.5–23.7, which in turn reacts with 3-OHP (eqn (23.9)).³⁶

It can be concluded that in the presence of HA as a sensitizer, 3-OHP is degraded by $^1\text{O}_2$, whereas Ph only reacts in its anionic form. On the other hand, under Rf + HA-photosensitization the species $^1\text{O}_2$ and $\text{O}_2^{\cdot-}$ are able to degrade 3-OHP.

The enhancement in the photodegradation rate exerted by the ionized species of ArSOH is a well-known effect in the reactivity of this family of substrates towards $^1\text{O}_2$.²

The generation of $\text{O}_2^{\cdot-}$ by direct electron transfer from $^3\text{Rf}^*$ to dissolved oxygen is an extremely low quantum yield process and can be neglected³¹ (process 23.3). Processes 23.2 could constitute a significant fraction of the overall quenching of $^3\text{Rf}^*$ by 3-OHP. Even when $\text{O}_2^{\cdot-}$ is not directly formed from the precursory species Rf^- , due to the fast protonation process, represented by reaction 23.5, superoxide radical anion could be in any case generated through step 23.7. The species RfH_2 is highly sensitive to traces of oxygen.³⁷

The production of the reactive species $\text{O}_2^{\cdot-}$ and $^1\text{O}_2$ can be competitive. The prevalence of each reactive channel would depend, in principle, on the relative kinetic weight of the respective energy-transfer and electron-transfer processes represented by reactions 23.4 and 23.11.

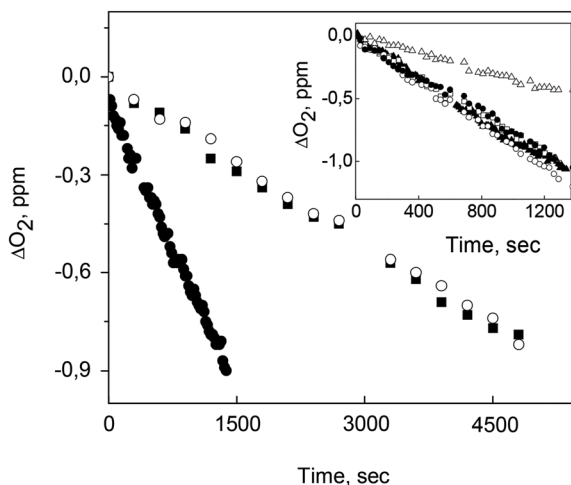


Figure 23.3. Oxygen consumption as a function of photoirradiation time for: a pH 7 aqueous solution of $50 \mu\text{g mL}^{-1}$ HA (○); the same in the presence of 0.5 mM phenol at pH 7 (■) and a pH 11 aqueous solution of $50 \mu\text{g mL}^{-1}$ HA plus 0.5 mM phenol (●). Inset: oxygen consumption as a function of photoirradiation time for a pH 7 aqueous solution of $50 \mu\text{g mL}^{-1}$ HA plus 0.5 mM 3-OHP (●). The same in the individual presence of: 0.5 mM NaN_3 (Δ); $1 \mu\text{g mL}^{-1}$ SOD (□); $1 \mu\text{g mL}^{-1}$ CAT (■); 10 mM mannitol (○). In all cases cut off at 360 nm. Reprinted from A. Pajares, M. Bregliani, J. Natera, S. Criado, S. Miskoski, J. P. Escalada and N. A. García, Mechanism of the photosensitizing action of a mixture humic acid-riboflavin in the degradation of water contaminants, *J. Photochem. Photobiol., A*, 2011, **219** (1), 84–89, Copyright 2011, with permission of Elsevier.

A kinetic analysis, based in the comparison of the respective rate-constant values helps in the mechanistic elucidation.^{38,39} It can be concluded that for the same concentrations of 3-OHP and dissolved O_2 , the rate for $\text{O}_2^{\cdot -}$ generation (*via* reactions 23.4–23.7, a process represented by reaction 23.7, is practically the same as the corresponding one for $^1\text{O}_2$ production from reaction 23.11.³⁶ As a consequence, both processes could simultaneously operate under work conditions.

Regarding HA, although this sensitizer is photodegraded with a moderate rate (Figure 23.3), it is continuously restored in natural waters through the microbial-mediated and the $^1\text{O}_2$ -mediated reactions described in Sections 23.1 and 23.3, respectively.

23.5. Final Remarks

ROS, including radical compounds and very specially $^1\text{O}_2$, participate in the GA-condensation/polymerization reactions, promoting the natural synthesis of HA in the presence of Rf. Both species are native photosensitizers of surface waters. The mixture HA + Rf photo-oxidizes common water contaminants, such as OHAr, *via* $^1\text{O}_2$ and $\text{O}_2^{\cdot -}$, under simulated environmental conditions.

In other words and within a more general frame, the pieces of experimental evidence here examined contribute to the characterization of essential photodegradation pathways in polluted waters. These facts demonstrate that: (a) photogenerated $^1\text{O}_2$ contributes to the generation of photosensitizers in nature and (b) the oxidative species represents a non-negligible input to environmentally friendly natural waters decontamination. In the case of contamination by pesticides, their use for specific purposes is conditioned by their persistence in nature. Hence, the design of photo-oxidizable pesticides, degradable under field conditions and with some degree of *predictable degradation time*, constitutes an interesting and important challenge.

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