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Practical Aspects in the Study of Biological Photosensitization Including Reaction Mechanisms and Product Analyses: A Do's and Don'ts Guide†

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ABSTRACT

The interaction of light with natural matter leads to a plethora of photosensitized reactions. These reactions cause the degradation of biomolecules, such as DNA, lipids, proteins, being therefore detrimental to the living organisms, or they can also be beneficial by allowing the treatment of several diseases by photomedicine. Based on the molecular mechanistic understanding of the photosensitization reactions, we propose to classify them in four processes: oxygen-dependent (type I and type II processes) and oxygen-*in*dependent [triplet-triplet energy transfer (TTET) and photoadduct formation]. In here, these processes are discussed by considering a wide variety of approaches including time-resolved and steady-state techniques, together with solvent, quencher, and scavenger effects. The main aim of this survey is to provide a description of general techniques and approaches that can be used to investigate photosensitization reactions of biomolecules together with basic recommendations on good practices. Illustration of the suitability of these approaches is provided by the measurement of key biomarkers of singlet oxygen and one-electron oxidation reactions in both isolated and cellular DNA. Our work is an educational review that is mostly addressed to students and beginners.

INTRODUCTION

Electromagnetic radiation induces chemical modifications in compounds present in living systems. When photons are directly absorbed by biomolecules, excited electronic states are generated, the compound becomes more reactive and, if the energy is not rapidly dissipated as heat or light, a photochemical reaction can take place. Alternatively, the photochemical change can occur indirectly through a photosensitized reaction, that is defined as a process by which a chemical change occurs in one compound, the substrate or target, as a result of the initial absorption of electromagnetic radiation by the photosensitizer (or referred to simply as sensitizer). Most of the solar energy incidence on Earth's surface corresponds to ultraviolet A (UVA) radiation (320–400 nm), visible (Vis) light (400–700 nm) and infrared (>700 nm) and is barely absorbed by the main biological chromophores. However, in particular UVA and Vis radiation can induce chemical changes in biological systems through photosensitized reactions (1–3), while relatively minor UVB component of solar radiation is predominantly responsible of the direct photochemical effects on several biomolecules.

Photosensitized processes produce beneficial and harmful effects in living organisms. UVA radiation is recognized as a class I carcinogen (4) and epidemiological evidence shows that exposure of humans to artificial UVA radiation (*i.e.* sun lamps and tanning beds) is a major risk factor for melanoma induction (5–7). However, photosensitization reactions have been applied in the development of important applications for the treatment of infections (photodynamic inactivation of microorganisms (PDI)) (8–11) and cancer (photodynamic therapy (PDT)) (12–14).

Endogenous photosensitizers are compounds, usually present at very low concentrations, which limits their capacity to cause photodamage, but they can accumulate under certain pathological situations. Many natural heterocyclic compounds, such as porphyrins, flavins, pterins and lumazines (15) and oxidation products of normal cellular components such as some oxidative degradation products of tryptophan (Trp) (16–18) can be considered as endogenous photosensitizers. Among exogenous photosensitizers a large number of xenobiotics can be included, incorporated to living organisms as pharmaceutical drugs or pollutants. In addition, many groups of compounds have been used as exogenous photosensitizers in PDI and PDT applications, such as porphyrins, chlorins, phenothiazines and furocoumarins (19–21). There are many novel techniques and methods of high current interest, such as optogenetic sensitizers (genetically-encoded for cell targeting), nanoparticles and liposomal carriers, plasmon-driven energy transfer, multifunctional and superhydrophobic sensitizers (22–25), that will also benefit from the concepts

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discussed in here, but that will not be further mentioned in detail, since we aim to discuss basic methodological and theoretical concepts.

Regarding the biological substrates, many biomolecules can undergo chemical modifications through photosensitized reactions. Taking into account their reactivity, concentration, and biological relevance, we will focus on the of main targets including some amino acids, i.e., tryptophan (Trp), tyrosine (Tyr), histidine (His), methionine (Met), cysteine (Cys), the nucleobases, mostly guanine (G) and to a lesser extent adenine (A), thymine (T), cytosine (C)), and the unsaturated fatty acids.

Due to the relevance in biology and medicine, the number of reports on photosensitization has been growing steadily in the last decades. Countless studies have dealt with a large number of photosensitizers, targets, molecular mechanisms and therapeutic applications and assessment of photodamage in different systems. Because of the diversity of issues investigated and the heterogeneity of the backgrounds of the researchers involved, many different approaches and methods have been developed and, therefore, valuable information in the scientific literature is sometimes scattered and difficult to sort. We have recently published a review article on biological photosensitization reactions that intends to unify definitions, sort the main mechanisms and gather relevant examples (26).

Fundamental research in photosensitization is essential to understand the molecular basis of these processes that affect living organisms. In particular, knowledge on mechanisms is crucial to improve strategies to avoid the harmful effects of electromagnetic radiation on biological system and to develop new photosensitizers and methods for PDI and PDT. With this in mind, we present here a guide to techniques with different levels of complexity to elucidate mechanisms of photosensitization processes. We also assess strategies for combining techniques and make some recommendations on good practices. This survey, by no means, includes detailed descriptions of techniques and is not an exhaustive report of the state-of-the-art approaches that are used to gain insights into photosensitization mechanisms, which can be found in the literature. In contrast to previous reviews (26,27), the present review focuses on practical aspects, including do's and don'ts in evaluating experimental data to acquire mechanistic information on photosensitized reactions. Consequently, this article should be considered as an educational review that is mostly addressed to students, beginners and non-specialists in the molecular fundamentals of photosensitization.

Mechanisms of photosensitization reactions

The well-known type I and type II mechanisms of photosensitized oxidations, initially defined by Foote (28) and more recently revisited (27), are mainly restricted to oxygen-dependent processes, in which molecular oxygen (O_2) is required in the process. In addition to type I and type II photosensitized oxidations, several mechanisms have been described for oxygen-independent processes. In our previous review article, we have proposed a simple and schematic classification of the most relevant mechanisms described in the literature together with representative examples (26). To provide context, in the next paragraphs the different types of mechanisms are summarized following the criteria used in Ref. (26) (Scheme 1).

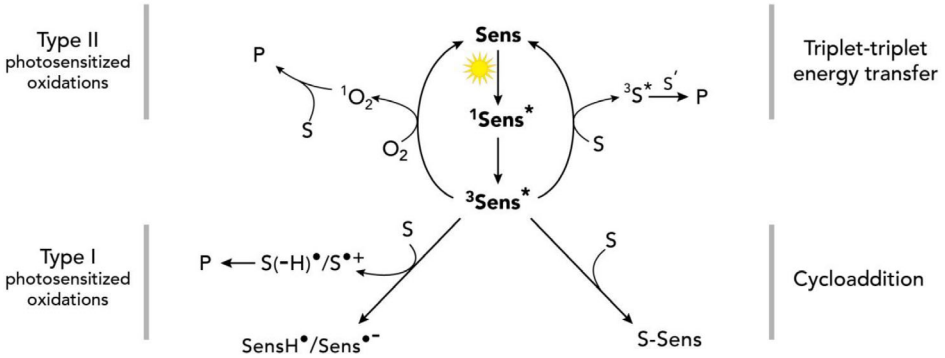
The first step in any mechanism is the absorption of a UV/Vis photon by the photosensitizer giving rise to a singlet excited state that usually undergoes intersystem crossing (ISC) to yield a longer lived triplet excited state (Scheme 1), which not always, but most of the time, is the reactive species responsible for the initiation of the chemical process. To understand this fact, some simple kinetic aspects must be considered and will be briefly discussed in the next section. Then the first bimolecular step of the mechanism is the reaction of the singlet or the triplet excited state of the sensitizer with the substrate or with O_2 (Scheme 1). If the photosensitizer reacts with the substrate, the process is contact dependent, namely, an encounter between the concerned species takes place. Many different types of bimolecular reactions need an encounter, such as electron transfer, hydrogen abstraction, processes that require orbital overlap, energy transfer taking place through space, *etc*. If this step is a dynamic process, the rate of encounters is controlled by diffusion and the involved sensitizer excited state is usually a triplet excited state. Intersystem crossing can be stimulated by charge-transfer and double spin-flip processes, while the quenching of the triplet excited state sensitizer by O_2 is a very frequent process leading to the formation of singlet oxygen ($^1O_2$) (see below) (29–33). In contrast, if there is a previous association between the photosensitizer and the substrate, the process that is not limited by diffusion, can be much faster and singlet excited state can also be involved.

In type I mechanisms, the first chemical reaction involves an electron transfer, a proton-coupled electron transfer or hydrogen atom transfer, leading to the formation of radicals. This initial bimolecular reaction can take place in both directions, but, almost always, the biomolecule target is the species that undergoes oxidation. In these processes, O_2 participates in subsequent reactions. Alternatively, the other first bimolecular reaction that can initiate a type I mechanism is the reduction of O_2 by the photosensitizer leading to the formation of superoxide anion radical ($O_2^{\bullet-}$). This mechanism is less important than the former and has not been included in Scheme 1.

Type II mechanism involves an initial energy transfer from the triplet excited state of the sensitizer to dissolved O_2, yielding singlet molecular oxygen [$O_2(^1\Delta_g)$, denoted throughout as $^1O_2$], the lowest excited state of O_2, which is generally far more reactive than ground state O_2 (34–39). Molecular orbital and spin-orbital diagrams of O_2 can be found in Refs. (37) and (40), as well as an educational undergraduate laboratory example of using $^1O_2$ for illustrating a [4 + 2] cycloaddition reaction in Ref. (41). Ground state O_2 is reactive on its own, including the redox reactions with metal ions, such as with Fe(II) compounds.

Oxidation by $^1O_2$ can be accomplished by using other methods of generation of this species different from classical type II photosensitization. It is worth mentioning few recently developed interesting technics, which will not be further discussed. Superhydrophobic sensitizers for the generation of airborne $^1O_2$ provide ways that avoids complications often found with sensitizers in biological matrixes (42). Singlet oxygen can also be generated in sensitizer-free systems by irradiation of O_2 itself at 765 nm (43), avoiding side reactions and other complications present when sensitizers interact with the diversity of molecules found in biological systems (44). However, the competing absorption by endogenous chromophores, the low extinction coefficient of O_2, and the need for femtosecond lasers can limit this direct-excitation technique in biological systems.

In oxygen-independent processes, the excited state photosensitizer reacts with the substrate (Scheme 1), while O_2 does not



Scheme 1. Main types of photosensitized reactions of biological targets. Sens: photosensitizer in the ground state, ^{1}Sens*: photosensitizer in the singlet excited state, ^{3}Sens*: photosensitizer in the triplet excited state, S: substrate or target molecule. For simplicity, only processes initiated by ^{3}Sens* are shown.

participate in any subsequent reaction. The first bimolecular step can be a triplet-triplet energy transfer (TTET) from the sensitizer to the substrate, which, in the excited state is more reactive and may react with a vicinal molecule. A second group of oxygen-independent reactions involves the formation of photoadducts in which the sensitizer and the substrate become covalently bound.

Basic kinetics

The triplet excited state of a photosensitizer invariably has lower energy than the corresponding singlet excited state, which can be interpreted in the frame of the Hund's rule, *i.e.* for a given electron configuration, the orbital occupancy with maximum multiplicity has the lowest energy. In spite of its lower energy, triplet excited state is, almost always, the pertinent reactive species that initiates the photosensitized dynamic processes. One may ask: Why of the two excited states, the one with lowest energy is the most important for the photosensitized process? The key to understand this fact does not lie in the energy assessment, but in a simple and fundamental kinetic analysis.

According to Kasha's rule, the more energetic singlet electronic-excited states (S2, S3, etc) relax non-radiatively to the lowest vibrational level of the first electronic singlet excited state (S1) from where photophysical and photochemical processes take place. Kashas's rule applies to the singlet and triplet excited states, but there are some exceptions that will not be commented in here (45). With these concepts in mind, consider the S1 state of a given photosensitizer (^{1}Sens*) in a solution free of O_2 and of any other reactant. The three fundamental competing pathways for the decay of ^{1}Sens* are listed in Scheme 2, together with their corresponding rates. One of these pathways is the intersystem crossing to yield a triplet excited state (^{3}Sens*).

Since the decay of a triplet excited state to the corresponding ground state is a spin forbidden transition, the rate of its decay is much lower than the rate of the singlet excited state decay and, consequently, the lifetime of the former is much longer than the latter (Scheme 2). Under continuous irradiation the sample quickly achieves the steady-state condition, at which there is no change in the concentrations of the reactive intermediates. Therefore, under these conditions $(d[^1\text{Sens}^*]/dt)_{SS} = 0$ and $(d[^3\text{Sens}^*]/dt)_{SS} = 0$, resulting in Eqs. (1) and (2) for the steady state conditions for the singlet and triplet excited state, respectively. The rate of formation of ^{1}Sens* per unit of volume is the photon flux absorbed by the photosensitizer divided by the volume of the sample ($q_{n,p}^{a,V}$). Therefore Eqs. (3) and (4) can be applied to calculate the concentrations of both excited states (Φ_T is the quantum yield of triplet excited state formation).

$$\left(d\left[^1\text{Sens}^*\right]/dt\right)_{SS} = 0 = q_{n,p}^{a,V} - \Sigma k_i^S \left[^1\text{Sens}^*\right] \quad (1)$$

$$\left(d\left[^3\text{Sens}^*\right]/dt\right)_{SS} = 0 = q_{n,p}^{a,V}\,\Phi_T - \Sigma k_i^T \left[^3\text{Sens}^*\right] \quad (2)$$

$$\left[^1 Sens^*\right] = \frac{q_{n,p}^{a,V}}{\sum k_i^S} \quad (3)$$

$$\left[^3 Sens^*\right] = \frac{q_{n,p}^{a,V}\;\Phi_T}{\sum k_i^T} \quad (4)$$

Within this simple kinetic analysis, it is easy to understand that the higher reactivity of triplet excited states is mainly due to their higher concentrations during the irradiation. The derivation of Eqs. (3) and (4) supposed an ideal situation where the sensitizer is alone in solution, but they clearly show that any additional molecule will react with the excited state in higher concentration, that is the triplet excited state.

The thermodynamic feasibility

A question may be asked: Is a certain compound capable of photosensitizing the degradation of a given biomolecule? To answer this question, the first point that has to be considered is if the potential photosensitizer absorbs UV–Vis photons and whether part of the singlet excited states generated is converted into triplet excited states. Triplet excited states, with longer lifetimes than singlet excited states, are most often times responsible for dynamic photosensitized processes (Scheme 1). An exception to this assumption can be the case in which there is a previous association between the photosensitizer and the substrate and then, since the time between absorption and reaction is shorter, singlet excited state can react directly. With this in mind we analyze the properties of triplet excited stated, knowing that in some particular cases the same considerations can be applied to singlet excited states.

The triplet excited state of a potential photosensitizer should present two properties: (1) lifetime (τ_T, Scheme 2) long enough to diffuse and collide with the target molecule (dynamic encounter) and (2) to have enough energy ($\Delta E_{0,0}$) to react with the substrate. τ_T can be calculated by laser flash photolysis (LFP) studies, whereas $\Delta E_{0,0}$ can be estimated by theoretical calculations or by determining the phosphorescence spectrum at low

Equation	Type of process	Step rate	$d[\text{Sens}^*]/dt$	τ
$^1\text{Sens}^* \longrightarrow {}^3\text{Sens}^*$	Intersystem crossing	$k_{ISC}^S\,[^1\text{Sens}^*]$	$\Sigma k_i^S\,[^1\text{Sens}^*]$	$1/\Sigma k_i^S$
$^1\text{Sens}^* \longrightarrow \text{Sens}$	Internal conversion	$k_{IC}^S\,[^1\text{Sens}^*]$		
$^1\text{Sens}^* \longrightarrow \text{Sens} + h\nu'$	Fluorescence	$k_F^S\,[^1\text{Sens}^*]$		
$^3\text{Sens}^* \longrightarrow \text{Sens}$	(back) Intersystem crossing	$k_{-ISC}^T\,[^3\text{Sens}^*]$	$\Sigma k_i^T\,[^3\text{Sens}^*]$	$1/\Sigma k_i^T$
$^3\text{Sens}^* \longrightarrow \text{Sens} + h\nu''$	Phosphorescence	$k_P^T\,[^3\text{Sens}^*]$		

$$\Sigma k_i^S >> \Sigma k_i^T \Rightarrow \tau^S << \tau^T \Rightarrow [^1\text{Sens}^*] << [^3\text{Sens}^*]$$

Scheme 2. Unimolecular deactivation pathways of singlet (^{1}Sens*) and triplet (^{3}Sens*) excited states of a photosensitizer. k_i: rate constant, d[Sens*]/d*t*: rate of excited state decay, τ: excited state life time, τ_S and τ_T: lifetimes of singlet and triplet excited states, respectively.

temperatures. An energy transfer will be possible if $\Delta E_{0,0}$ is higher than the energy gap between the ground state and the excited state of the acceptor. However, it is worth mentioning that when the energy gap is large, other processes can compete, thereby reducing rates. Therefore, simple comparisons allow to assess the thermodynamic feasibility of a given process, that is, to find out if an electronically excited photosensitizer is able to transfer its energy to O_2 for generating $^1O_2$ (type II mechanism) or directly to a substrate to promote its conversion to an triplet excited state (TTET).

The analysis of thermodynamics of type I mechanisms is more complex. Considering the first bimolecular step as a redox reaction in which the substrate is oxidized (Scheme 1), the tendency of a photosensitizer in its electronic excited state to act as an oxidant can be assessed in terms of its one-electron reduction potential (E(Sens*/Sens$^{\bullet-}$)), also named as E'_{ox}. E(Sens*/Sens$^{\bullet-}$) can be estimated from the values of the one-electron reduction potential of the sensitizer in its ground state (E(Sens/Sens$^{\bullet-}$)) and $\Delta E_{0,0}$, expressed in electron-volts (eV) (Eq. 5). Then, the thermodynamic feasibility of the redox reaction can be evaluated by estimating the free energy change (ΔG) of the process (Eq. 6). Here, besides the E(Sens*/Sens$^{\bullet-}$) value, two additional parameters are needed: (1) the one-electron oxidation potential of the substrate (the electron donor), or better said, the reduction potential of the substrate radical, E(S$^{\bullet+}$/S), and (2) the energy required for the encounter of the reactants, that is, for bringing the reactant molecules together (Δw) (46,47). Δw value is related to the solvation energy of an ion pair Sens^{-}/S$^{\bullet+}$ and is very small compared to the one-electron potentials and, in most cases, can be ignored for strong polar solvents, such as H_2O.

$$E(\text{Sens}^*/\text{Sens}^{\bullet-}) = E(\text{Sens}/\text{Sens}^{\bullet-}) + \Delta E_{0,0}(\text{eV}) \quad (5)$$

$$\Delta G(\text{eV}) = E(\text{S}^{\bullet+}/\text{S}) - E(\text{Sens}^*/\text{Sens}^{\bullet-}) + \Delta w\ (\text{eV}) \quad (6)$$

Sometimes the information to assess the thermodynamic feasibility of a given process has been gained and is available in the literature; in other situations this is not the case and the values have to be theoretically or experimentally determined. It is important to take into account that thermodynamic analysis is just a first approach: if a given process is not thermodynamically possible, it can be discarded; on the other hand, if the process is feasible, this does not mean that the process is going to take place because kinetics have also to be considered. That is, a process, thermodynamically favored, might not occur because its rate is very low. Sometimes given a pair photosensitizer-substrate, several mechanisms are thermodynamically feasible and, in this case, those mechanisms will take place simultaneously and therefore compete, the predominant being the faster.

STEADY-STATE PHOTOLYSIS

Valuable information about the mechanism(s) involved in given photosensitization processes can be obtained by continuous or steady-state photolysis studies. In these experiments a sample containing the photosensitizer and the substrate is exposed to continuous electromagnetic radiation, using a source that emits in a range of wavelengths for which the photosensitizer absorbs whereas the substrate does not. In this way, excited states are generated only in the photosensitizer and if the substrate undergoes any chemical change, it will be due to photosensitization. The experiment must be carried out in a receptacle with transparent walls, for instance a quartz cell, and under controlled conditions, such as defined reactants concentrations, temperature, pH, *etc*. After irradiation the concentrations of both reactants are determined, using a suitable technique, typically, chromatography or spectroscopic analysis.

A first experimental approach

Comparative photolysis in the presence and the absence of O_2 can be a first experimental approach to find out the type of mechanism involved in a given photosensitization process. The concentration of O_2 depends on several factors, including the temperature, the pressure and concentration of other compounds present in solution. O_2's solubility in liquids at constant pressure and temperature and under equilibrium conditions is usually 10 times larger in organic solvents compared to that of water (48,49). The more apolar the solvent, the higher the O_2 solubility (50). O_2 can be easily removed from solvents by bubbling argon or nitrogen in the solution, or more efficiently by freeze-pump-thaw (51). The reduction of O_2 concentration depends on many factors, including the expertise of the researcher performing the degassing, but it can be as high as 40 times (49). One practical aspect is to achieve truly O_2 free conditions: freeze–thaw-degassing that is required for this purpose for solutions is not compatible for cells. Another approach for O_2 removal associates type II photosensitization and chemical trapping, thus attaining even lower O_2 concentrations (52). It is also worth mentioning that the suppression of the emission by tris(2,2′-bipyridine)

ruthenium(II) ($Ru(bpy)_3$) provides an efficient and clean method to detect O_2 concentration in solution (53). The O_2 concentration is expected to cause significant changes in the kinetics and yields of products, unless triplet excited states are not taking part in the investigated photochemical process. In this case, either the photochemical reactions are initiated by singlet excited states or other species that compete with the free photosensitizer, such as the formation of dimers, aggregates or non-covalent complexes with biological species that inhibit the O_2/excited-photosensitizer encounter (see further discussions below) (54,55).

The pattern of photosensitizer degradation, which is also named photobleaching, and the consumption of substrate upon irradiation will be different for the various types of mechanisms. Both type I and type II photooxidation mechanisms need O_2 and the photosensitizer is not consumed in the process. That is true for model and simplified mechanisms, such as those described below in Eqs. (7–11). However, in type II mechanisms, the photosensitizer many times can be consumed by the reaction with $^1O_2$. In addition, if the radical anion derived from the photosensitizer is not completely re-oxidized some consumption can be observed in type I mechanisms. This is particularly relevant in photooxidation of lipid membranes (56). In general, for photosensitized oxidations, even in cases that deviate from ideal mechanisms, the consumption of the photosensitizer is significantly lower than that of the substrate and O_2. In this context, methods that are photosensitizer free (43) or use phase-separated photosensitizer, such as superhydrophobic materials (42,57) can be advantageous.

In oxygen-independent processes, photosensitization takes place under anaerobic conditions and in air-equilibrated solutions the substrate consumption is, in general, slower because O_2 competes with the substrate for the triplet excited state of the photosensitizer. For the formation of photo-adducts the photosensitizer is always consumed and the relation between the consumption of both reactants is expected to be 1:1. In contrast, in TTET mechanisms, the photosensitizer in its ground state is recovered upon reaction with the substrate and, in consequence, no consumption of the photosensitizer is expected. Table 1 schematically shows the pattern of consumption of the photosensitizer and substrate for each type of mechanisms. Of course, this analysis is a simplification that does not include some exceptions and (frequent) cases in which more than one mechanism is occurring simultaneously.

However, even when it is not expected (Table 1), often, a decrease in the concentration of the photosensitizer is observed upon irradiation of a solution containing both reactants. The photobleaching is not necessarily due to the same process in which the photosensitizer is causing the degradation of the substrate. In contrast, the photosensitizer can be photo-unstable, undergoing intrinsic photolysis upon light exposure. For this reason, this type of experiments must be accompanied by controls in which a solution of the photosensitizer is irradiated in the absence of substrate. The irradiation of the substrate in the absence of sensitizer is also an important control because it ensures that the substrate does not undergoe direct photodegradation and the consumption in the solutions containing both reactants is due to photosensitization.

Table 1. Pattern of consumption of photosensitizer and substrate for different types of mechanisms. These findings are valid only when a unique mechanism is possible for a given pair sensitizer/substrate.

		Photosensitized oxidations		O_2 independent processes	
	Reactant	Type I	Type II	TTET	Photoadducts
Presence of O_2	Sensitizer	−/+	−	−	+
	Substrate	+	+	+	+
Absence of O_2	Sensitizer	−	−	−	++
	Substrate	−	−	++	++

TTET = triplet-triplet energy transfer.

Another interesting experimental consideration during the photosensitized process is the evaluation of O_2 consumption, by determining its concentration before and after irradiation of the solution containing the photosensitizer and the substrate. O_2-selective electrodes can be used for monitoring the O_2 concentration upon irradiation of solutions initially saturated with air. Again, comparative controls carried out with solutions containing only either the substrate or the sensitizer are mandatory for a clear interpretation of the results. If the consumption of O_2 is faster than in the controls, a photosensitized oxidation (type I and/or type II mechanisms) is taking place.

Type I *versus* type II mechanisms

PDT and PDI are based on the appropriate combination of electromagnetic radiation, a photosensitizer and O_2 to kill cells (cancer cell or pathogenic microorganism). Therefore, the term "photodynamic" involves photosensitized oxidations. In some applications, like in cancer treatment, a suitable level of tissue oxygenation is not easy to obtain, a difficulty that is considered to be responsible for most of the PDT failures in cancer treatment, since most solid tumors are characterized by low O_2 concentration (58). The role of O_2 concentration becomes evident in the treatment of the diabetic foot for patients suffering from periphery arterial disease (PAD). Cohort studies and the current accepted prediction algorithms indicate that the treatment of the diabetic foot will not be effective, unless tissue revascularization is performed in patients with PAD (59). The search for photosensitizers that perform properly at low O_2 concentration is a hot-topic in PDT research, but no oxygen-independent photosensitizer has been tested in humans (60).

If we take into account that generation of skin cancer and other types of photodamage to living organisms also involves the participation of O_2, it became evident that photosensitized oxidations plays a pivotal role in skin diseases. Frequently, given a pair photosensitizer/substrate, type I and type II mechanisms are both thermodynamically feasible and compete while dependent on O_2 concentration (see Reactions 7–12). In these cases, the predominant mechanism is not easy to establish and many times would depend not only on the reactants, but also on the experimental conditions. Anyhow, we must consider whether we can learn anything about the photosensitization oxidation mechanism(s) in well-controlled experimental setup, by assessing changes that are dependent on O_2 concentration. In this section we will present analysis of increasing complexity to get information about the predominant mechanism in a given photosensitized process.

Two photosensitized oxidations may be considered: The first one (process 1) takes place exclusively through type I mechanism while process 2 occurs *via* a pure type II mechanism. Process 1 can be a case in which the substrate undergoes one-electron oxidation by the triplet excited state of the photosensitizer, but it does not react with $^1O_2$. Process 2 can be a case in which the ΔG value of the redox reaction is positive (Eq. 6), but the photosensitizer is able to generate $^1O_2$, which, in turn, oxidizes the substrate.

Next, one should take into account Reactions 7–9 that schematically represent the main steps of type I mechanism and Reactions 10 and 11 that schematically represent the main steps of type II mechanism. It is worth mentioning that sometimes O_2 deactivates the triplet excited states without generating $^1O_2$ (Reaction 12). This is the main reason why the rate of quenching of the photosensitizer triplet excited states may be different from the rate of $^1O_2$ formation.

$$^3\text{Sens}^* + \text{S} \rightarrow \text{Sens}^{\bullet-}/\text{SensH}^\bullet + \text{S}^{\bullet+}/\text{S}(-\text{H})^\bullet \quad (7)$$

$$\text{Sens}^{\bullet-}/\text{SensH}^\bullet + \text{O}_2 \rightarrow \text{Sens} + \text{O}_2^{\bullet-}/\text{HO}_2^\bullet \quad (8)$$

$$\text{S}^{\bullet+}/\text{S}(-\text{H})^\bullet \xrightarrow{\text{O}_2/\text{O}_2^{\bullet-}/\text{H}_2\text{O}} \text{S}_{(\text{ox})} \quad (9)$$

$$^3\text{Sens}^* + \text{O}_2 \rightarrow \text{Sens} + {}^1\text{O}_2 \quad (10)$$

$$\text{S} + {}^1\text{O}_2 \rightarrow \text{S}_{(\text{ox})} \quad (11)$$

$$^3\text{Sens}^* + \text{O}_2 \rightarrow \text{Sens} + \text{O}_2 \quad (12)$$

The quantum yield of $^1O_2$ production (Φ_Δ) is given by Eq. (13),

$$\Phi_\Delta = \Phi_T \, \phi_{et} \quad (13)$$

where ϕ_{et} is the efficiency of energy transfer from the triplet excited state of the photosensitizer to O_2, that is the fraction of triplet excited state that is quenched in Reaction 10 (Eq. 14).

$$\phi_{et} = \frac{k_{et}}{\Sigma k_i^T + k_S^T[S] + k_{O_2}^T[O_2]} \quad (14)$$

In Eq. (14), Σk_i^T is the sum of the rate constants of the unimolecular deactivation pathways (Scheme 2), k_S^T is the rate constant of the quenching of triplet excited state by the substrate and $k_{O_2}^T$ is the rate constant of the quenching of triplet excited state by O_2, in which both Reactions 10 and 12 contribute.

With these pathways and kinetic analysis in mind, it is possible to explain the different behavior observed for the consumption of the substrate as a function of irradiation time in photolysis experiments carried out under various conditions. The increase in the concentration of O_2, easily accomplished by saturating the solution with the gas before irradiation, will always favor the type II mechanism because the rate of Reaction 10 depends directly on O_2 concentration. Therefore, for process 2 the rate of substrate consumption will be higher in O_2–saturated than in air-equilibrated solutions (Fig. 1). This difference will be more or less significant depending on the fraction of the triplet excited state of the sensitizer quenched under both O_2 concentration conditions. On the other hand, the increase in O_2 concentration will hinder a type I mechanism because O_2 does not participate in Reaction 7 and consumes part of the triplet excited states of the sensitizer through Reactions 10 and 12. Therefore, for process 1 the rate of substrate consumption may be lower in O_2–saturated than in air-equilibrated solutions (Fig. 1).

Besides the concentration of O_2, other variables, such as changing the solvent may bring important information concerning the photochemical mechanism. The lifetime of $^1O_2$ (τ_Δ) in D_2O is much longer than in H_2O, because protic solvents lead to the short τ_Δ due to facile deactivation by O–H vibrational quenching to ground state $^3O_2$ (61). Therefore, the photosensitized oxidation of the substrate will be much faster in D_2O than in H_2O if $^1O_2$ contributes significantly to the process. In cells, the complete exchange of H_2O for D_2O cannot be achieved, since some residual H_2O always remains, so the exact τ_Δ increase from D_2O is more

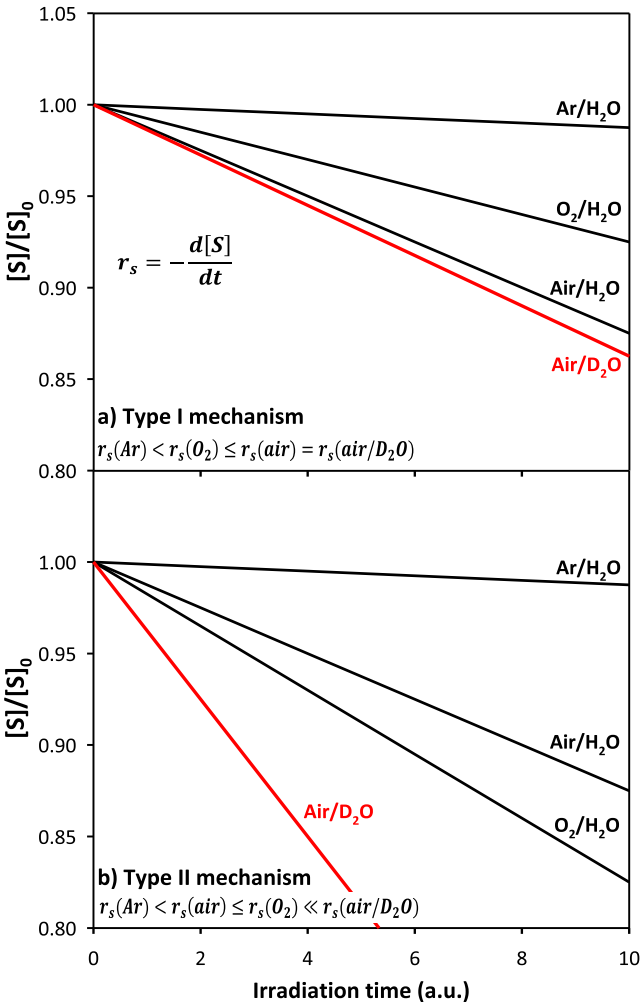


Figure 1. Typical behavior of the time evolution of the concentration of an oxidizable substrate (S) steadily irradiated in the presence of a suitable photosensitizer under different experimental conditions. (a) Process 1 takes place through a pure type I mechanism; (b) process 2 takes place through a pure type II mechanism.

qualitative than quantitative. In contrast, if $^1O_2$ does not participate at all in the mechanism, the rate of substrate consumption will be similar in both solvents. This behavior is schematically shown for the hypothetic processes 1 and 2 in Fig. 1.

Regarding Fig. 1 a relevant observation is necessary. The linear behavior depicted for all the cases presented in Fig. 1 is just for the sake of simplicity and lacks from scientific thoroughness. In the type of experiments proposed in this section, the linear plot is a particular case under specific conditions, such as short periods of irradiation, within which the consumption of the reactant (substrate) is very small, for instance lower than 15%. The general case is curvature (concave up) because the significant consumption of the substrate makes the reaction slower, that is, the absolute value or modulus of the slope of the curve decreases with irradiation time.

Kinetic assessment of the contribution of type II mechanism

In solution, $^1O_2$ relaxes to its ground state through solvent induced radiationless and radiative pathways (Reactions 15 and 16). Alternatively, it may participate in bimolecular processes with

other substances present in the medium. In this way, $^1O_2$ may be physically deactivated by the substrate (Reaction 17) and/or can oxidize it (Reaction 18, Reaction 11 of the previous section) (62). The same reactions can take place with the photosensitizer (Reactions 19 and 20). Reaction 20, if it takes place, will lead to the photodegradation of the sensitizer, and will be evidenced in the control carried out in the absence of the substrate.

$$^1O_2 \rightarrow {}^3O_2 \quad (15)$$

$$^1O_2 \rightarrow {}^3O_2 + h\nu \quad (16)$$

$$S + {}^1O_2 \rightarrow S + {}^3O_2 \quad (17)$$

$$S + {}^1O_2 \rightarrow S_{(ox)} \quad (18)$$

$$Sens + {}^1O_2 \rightarrow Sens + {}^3O_2 \quad (19)$$

$$Sens + {}^1O_2 \rightarrow S_{(ox)} \quad (20)$$

The rate of the oxidation of a defined substrate by $^1O_2$ (Reaction 18) is given by Eq. 21:

$$(d[S]/dt)_\Delta = -k^\Delta_{r-S}\left[{}^1O_2\right][S] \quad (21)$$

where k^Δ_{r-S} is the rate constant of the chemical reaction between $^1O_2$ and the substrate. If a solution containing the substrate and the photosensitizer is continuously irradiated at either a wavelength or a range of wavelengths absorbed only by the photosensitizer, the rate of $^1O_2$ production is given by Eq. (22),

$$r_\Delta = q^{a,V}_{n,p}\,\Phi_\Delta \quad (22)$$

where $q^{a,V}_{n,p}$ and Φ_Δ are the photon flux absorbed by the photosensitizer and its quantum yield of $^1O_2$ production, respectively; and the rate of $^1O_2$ consumption is given by the sum of the rates of the Reactions 15–20 (Eq. 23),

$$r_{-\Delta} = -\left(k_d\left[{}^1O_2\right] + k^\Delta_{t-Sens}[Sens]\left[{}^1O_2\right] + k^\Delta_{t-S}[S]\left[{}^1O_2\right]\right) \quad (23)$$

where k_d is the non-radiative deactivation rate constant (Reaction 15) (61) [k_e, the radiative deactivation rate constant (Reaction 16), is negligible compared to k_d (63,64)]; k^Δ_{t-S} is the rate constant of $^1O_2$ total quenching by the target molecule and is the sum of k^Δ_{r-S} (Reaction 18) and the rate constant of the physical quenching of $^1O_2$ by the substrate (k^Δ_{p-S}) (Reaction 20) (Eq. 24); similarly, k^Δ_{t-Sens} is the rate constant of $^1O_2$ total quenching by the photosensitizer.

$$k^\Delta_{t-S} = k^\Delta_{r-S} + k^\Delta_{p-S} \quad (24)$$

Assuming steady-state conditions, that is to say, the rates of $^1O_2$ formation and consumption are equal (Eq. 25), the steady-state concentration of $^1O_2$ can be estimated with Eq. 26.

$$r_\Delta + r_{-\Delta} = 0 \quad (25)$$

$$\left[{}^1O_2\right] = \frac{q^{a,V}_{n,p}\,\Phi_\Delta}{k_d + k^\Delta_{t-Sens}[Sens] + k^\Delta_{t-S}[S]} \quad (26)$$

For a given set of conditions (irradiation intensity, concentrations, etc), the experimental rate of substrate consumption ($(d[S]/dt)_{exp}$), determined, for instance, by chromatographic analysis, is compared to $(d[S]/dt)_\Delta$ (Eq. 21). If $(d[S]/dt)_{exp}$ is similar to $(d[S]/dt)_\Delta$, the predominance of a $^1O_2$–mediated mechanism can be inferred. In contrast, if $(d[S]/dt)_\Delta$ is much lower than $(d[S]/dt)_{exp}$, it can be assumed that oxidation *via* type II mechanism represents a minor contribution.

This kinetic analysis allows a reliable method to assess the contribution of type II mechanism to the overall photosensitized oxidation of a substrate (65) and is much more accurate than the rough analysis presented in the previous section. The main difficulty of this method is that all parameters of Eqs. (22) and (26) have to be determined. In particular, k^Δ_{t-Sens}, k^Δ_{t-S} and $q^{a,V}_{n,p}$ values are difficult to measure and require a significant experimental effort.

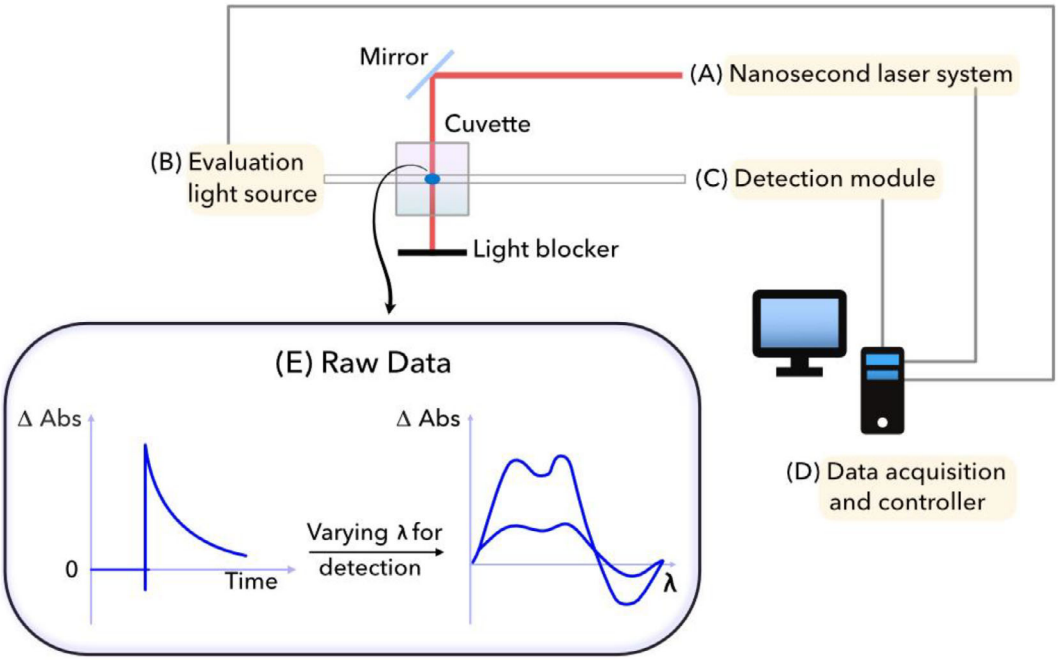
DETECTION OF REACTIVE INTERMEDIATES

Laser flash photolysis (LFP): Intermediates triplets and radicals

One of the most useful tools to evaluate photosensitized oxidation processes is to assess the intermediate species in solution after a laser pulse, by a method called laser flash photolysis (LFP), a type of pump-probe spectroscopy that provides information on intermediates within time scales ranging from femtoseconds up to micro and milliseconds. The reactive intermediates relevant to photosensitized oxidations are generated, diffuse and react on this time scale, making LFP a flexible and fundamental method to investigate the mechanisms taking place during light-induced oxidations (66), as well as, the dynamics of interactions and complexation (67).

Although many types of apparatus and configurations have been developed and used, some common and relevant aspects can be mentioned (Scheme 3). The sample excitation is often performed by a nanosecond laser system, such as a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser coupled or not with optical parametric oscillator (OPO). Older technology also included coupling with dye laser. The sample solution should have absorbance in the excitation wavelength (0.1–0.3 absorbance units) to stave off spectral problems such as inner filter effects. The light beam for acquiring the transient absorption can be from a continuous light source like a Xe lamp (usually wavelength selected) or from a LED. In this case, the pulse of the evaluation source is controlled to start before the laser excitation pulse. However, currently continuous wave (CW) LED and Xe lamp systems are more frequently used. The detection system often includes a monochromator or a photomultiplier tube, or charge-coupled device. Software controls the timing of the laser and evaluation light pulses, as well as the opening of the acquisition shutter and the signal acquisition. Raw data is in the form of transient signals of the absorbance variations (before and after the laser pulse, ΔAbs) that could be negative or positive, depending on whether the transient species absorbs more or less than the ground state in the observed wavelength. By varying the wavelength of data acquisition in systems equipped with a monochromator, it is possible to obtain transient spectra.

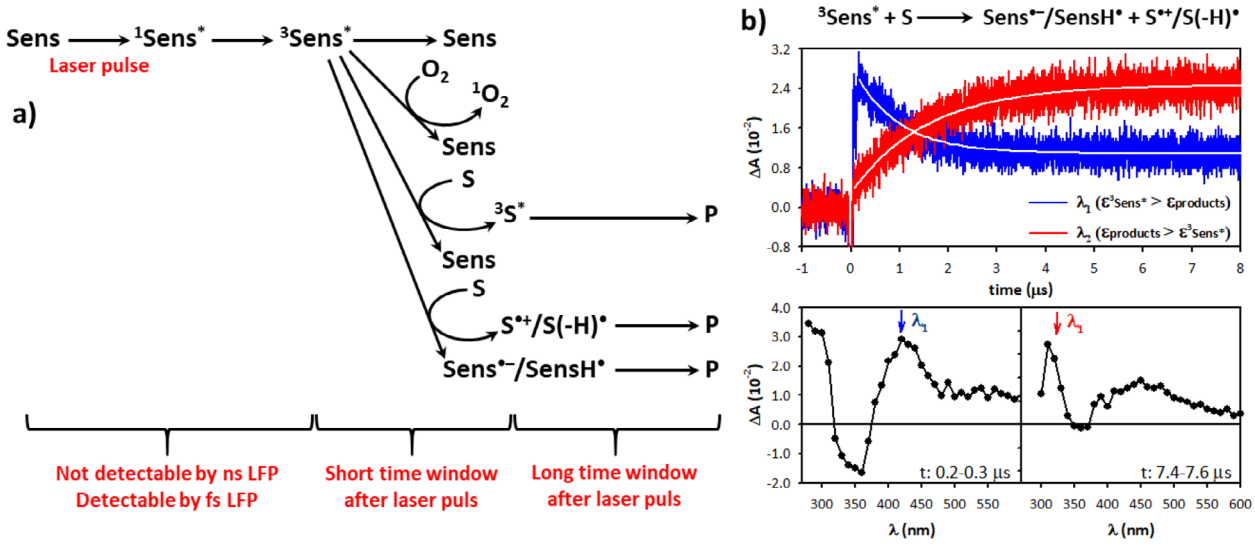
The transient absorption obtained after a laser flash (pump) can be used to study the characteristics and reactivity (obviously both properties are connected) of the intermediate species. In general, the formation of triplet excited state cannot be detected by the more common apparatus that uses nanosecond laser pulse, but can be investigated with femtosecond LFP systems. The following steps, reactions of triplet excited with O_2 or the substrate, can be monitored for all LFP equipment (Scheme 4a). Usually, the initial signals after the laser pulse are attributed either to triplet excited states or to radical species. For example, the initial step in type I mechanism (Reaction 7) can typically be followed in the microsecond time domain (Scheme 4a), where both the decay of the triplet excited state of the photosensitizer or the formation of the radical can be monitored selecting suitable



Scheme 3. Schematic of a laser flash photolysis (LFP) setup and data acquisition. (A) Pulsed-laser irradiation; (B) pulsed-light source for evaluation of transient intermediate; (C) detection module, commonly a shutter, a monochromator and a photomultiplier tube (PMT) coupled to an oscilloscope; (D) software for timing control of the laser, evaluation light source, shutter, and signal acquisition; and (E) raw data as transient signals with absorbance variations, before and after the laser pulses (ΔAbs).

wavelengths (Scheme 4b) (68). Moreover, the transient spectra recorded at short times after the laser pulse correspond to the former species, whereas those recorded at long times correspond to the latter (Scheme 4b). Following the transient at longer time scales (typically, from 100 μs to milliseconds) (Scheme 4a), may provide hint of secondary and higher order intermediates, as well as, information on final products.

Decreasing O_2 concentration could be used to characterize the chemical identity of the intermediate species. A triplet excited state will have its lifetime increased by at least one order of magnitude when O_2 is removed, since O_2 suppresses most triplet excited states (Reactions 10 and 12). Unfortunately, anion or semi-reduced radicals will also have their lifetimes increased in the absence of O_2, since O_2 usually re-oxide these radicals, forming $O_2^{\bullet-}$ (Reaction 8). Other experiments have to be performed in order to identify the nature of the intermediate whose lifetime increases in the absence of O_2. For example, triplets are usually efficiently suppressed by carotenoids, while reduced radicals not necessarily (69). The redox properties of the radicals can be accessed by measuring their reactivity with molecules having different reduction



Scheme 4. (a) Portions of the laser flash photolysis (LFP) system involving: pulsed-excitation of sensitizer (Sens); intersystem crossing of singlet-excited sensitizer (^{1}Sens*) to triplet-excited sensitizer (^{3}Sens*); quenching of ^{3}Sens* in short-time window; reactions proceeding after long-time windows following the laser pulse. (b) LFP analysis of the initial step of type I mechanism. Representative signal traces and absorption spectra following ^{3}Sens* decay and transient intermediate appearance of transient intermediates: sensitizer and substrate radicals ($Sens^{\bullet-}$/$SensH^{\bullet}$ and $S^{\bullet+}$/$S(-H)^{\bullet}$, respectively).

potentials. This type of experiment can be used to characterize the range or redox properties of the radical species (56,70).

Can we learn from LFP transient signals that do not depend on the O_2 concentration? Although specific details need to be investigated in a case-by-case matter, one may consider at least three most frequent situations:

1 The triplet excited state is interacting with complexes that hinder the O_2 diffusion. Ground state O_2 is small, uncharged and readily diffuses in the condensed phase, and can react for example with proteins such as hemoglobin or redox active metal-containing compounds, but otherwise has a high energy barrier to activation. The packing density in a globular protein is very high and usually hinders the diffusion of O_2 (71). The protein conformation usually leads to pockets that can interact with the photosensitizer. A photosensitizer will typically react by electron transfer with specific redox-active biological targets, for example, amino acid components of proteins (72). The delivery of photosensitizers either bound to proteins or preferentially interacting with plasma proteins could be an issue for new generation photosensitizers (14,73).
2 Triplet excited states typically have lifetimes of nanoseconds to tens of microsecond in the biological environment, mainly because of the reaction with O_2. Triplet excited states have diffusion-limited reactions (or close to the diffusion limit) with O_2 (74). Excited states diffuse and react during their lifetimes. To estimate the sphere of activity of a triplet excited state, we propose to use the boundary conditions suggested by Ogilby, that assume a radial diffusion of the excited state and estimate the period at which the excited state is reactive (t) to be equal to 5 times the value of the excited state lifetime (τ) (39). Remember that during one lifetime the population of an excited state is reduced by a factor of $1/e$, and therefore, during 5 lifetimes the excited state population is reduced to <1% of the initial value (~0.67%). Taking a typical τ_T value (Scheme 2) of ~500 ns, the period of reaction of the triplet excited state will be around 2500 ns. For a molecule of molecular weight 300–400 g mol^{-1} and having diffusion coefficient (D) of ~3×10^{-6} cm^2 s^{-1}, its travel distance ($\delta = \sqrt{6Dt}$) will be around ~66 nm (75). Note, this travel distance is about 10 times smaller than that of $^1O_2$ (see below). Therefore, other reactions with the triplet excited states can take place in this limited time/spatial range. This usually occurs when the triplet excited state is interacting with the biological target in the ground state. The reaction will usually involve an electron transfer step with the biological target, which is an electron donor group such as a double bond of a lipid (56,76) amino acid (77) or a nitrogenous DNA base (78). Please note that this is a simple calculation to provide generic and rough estimations of lifetimes and diffusion distances. However, microheterogeneous concentrations may greatly affect this estimation. For example, ground state O_2 is small, and its concentration is ~10-fold higher in lipophilic than aqueous media. The former coincides with photosensitizer localization when found in biomembranes.
3 The photosensitizer is associated with itself in the ground state, forming dimers or higher order aggregates, which can facilitate excited-state quenching or lead to chemical reactions. An intermolecular electron transfer reaction will form the semi-oxidized and the semi-reduced forms of the photosensitizer typically on picosecond time scales (54,79,80).

Singlet oxygen phosphorescence in the NIR

Near-infrared (NIR) luminescence emission centered at 1270 nm, which is the fingerprint emission of the $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ transition, is the most specific way to detect the generation and study the reactivity of $^1O_2$, and consequently to characterize the role of type II photosensitized oxidation reaction. This method is not very sensitive because the intrinsic emission efficiency of $^1O_2$ is ultra-weak [only 1 in 10^6 of $^1O_2$ molecules luminesces (Reaction 16)]. Also the detectors available in this NIR are not as efficient as those used in the visible spectral range and the emission decay of $^1O_2$ occurs in the microsecond time domain, in such a way that emission, reaction and diffusion are in the same time domain. However, in this spectral region emission from few other molecules contributes, which leads to a very specific detection for $^1O_2$. In addition, NIR detectors have evolved and are more sensitive nowadays. This method has been at the origin of many discoveries in this field (81–83). As in any other emissive technique, it is possible to perform steady-state measurements to obtain emission spectra or to operate in the time-resolved mode for gaining kinetic information on the decay and reactivity of the excited transient (84). Interesting to mention that Gorman and Rodgers predicted, in this review (84), that the emissive characteristics of $^1O_2$ would prevent the kinetic analysis of its decay in complex environments, which proved to a wrong prediction, since kinetic analysis of $^1O_2$ decays could be detected in several complex scenarios, including single cells (see further discussion below).

The first aspect that should be considered in any method aiming to quantify $^1O_2$ is that τ_Δ varies quite substantially with the solvent, for example, from 3.7 μs in H_2O to 66 μs in D_2O and to almost 28 ms in CCl_4 (85–87). This is because solvent molecules interact and perturb $^1O_2$ both by charge transfer states and vibrational modes that mediate non-radiative coupling of $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ transition (88). Therefore, if one would like to compare yields of $^1O_2$ or its reactivity, it is imperative to consider the effect of the solvents, ideally comparing measurements performed in the same solvent (89). It is worth mentioning that the intrinsic τ_Δ value found in the literature for certain solvents varies quite substantially. This is because τ_Δ depends on the solvent purity, especially concerning small amounts of water or of other impurities. In solvents with longer intrinsic τ_Δ, or in complex environments (biological or materials) with high concentrations of photosensitizer and suppressors (quenchers), one should also worry with the suppression by the photosensitizer and by O_2. Besides being the main reactants during the $^1O_2$ generation, photosensitizer and O_2 may also act as suppressors themselves (61,90–93).

Taking into account the τ_Δ value and its diffusion coefficient, it is possible to estimate its average travel distance ($\delta = \sqrt{Dt}$) to be around ~660 nm in water ($D = 5 \times 10^{-5}$ cm^2 s^{-1}, τ_Δ ~ 3 μs, t = 15 μs) (39,94). The physical characteristics of $^1O_2$ (size, charge, polarity) are very similar to those of O_2, indicating the difficulty to restrain its distribution among different compartments. In terms of biological ultra-structural organization, 660 nm is not small since a membrane bilayer is only 5 nm thick and several organelles have sizes on the same order of magnitude than the diffusion distance of $^1O_2$. Consequently, many efforts have been devoted to the study of the diffusion and reaction of $^1O_2$ in higher complexity systems of interfaces, micelles, vesicles and cells. By preparing micellar systems in which the generation

of $^1O_2$ occurred in the bulk solution, while $^1O_2$ trapping was inside aqueous micelles of different charges, Gorman and colleagues showed that $^1O_2$ diffuses and reacts in micellar solutions similarly than in bulk water, *i.e.* $^1O_2$ is not affected by the characteristics of the water-organic interfaces (95). Studies in reverse micelles (water-in-oil nano-emulsions) resulted in similar observations. The NIR decay data obtained with photosensitizers dissolved in different reverse micelles could be fitted by considering that $^1O_2$ quickly diffuses and equilibrates in the micro-heterogeneous systems, without any measurable interference of the different interfaces (96). Therefore, in heterogeneous or micro-heterogeneous systems, for example in water-hydrocarbon domains, $^1O_2$ will have enough time to distribute between different phases and equilibrate following the tendency given by the partition coefficient. Therefore, considering the concentration gradients, one can consider that $^1O_2$ will be compartmentalized following the physical–chemical partition coefficients (97).

What is the situation when $^1O_2$ is generated within a membrane bilayer by membrane-embedded photosensitizers? To understand the NIR luminescence profiles in this condition one must consider the O_2 diffusion and partition as well as the luminescence rate constants in the membrane and water (84). In the membranes, $^1O_2$ has a higher luminescence rate constant and an environment that favor longer lifetimes, compared with water (98). However, the lipid bilayers are so thin compared with the diffusion radius of $^1O_2$ (see comments above) that $^1O_2$ exits from the membrane very rapidly. Therefore, NIR emission transients from liposome suspensions, in which the generation of $^1O_2$ occurs inside the membrane, will not bring information concerning the τ_Δ value in the environment of the lipid bilayer. The luminescence transient will show mainly the typical τ_Δ value observed in the bulk solution and, at very short times after the laser pulse, the average escaping time of $^1O_2$ from within the membrane (99). Therefore, $^1O_2$ molecules escape the membranes before they can actually "sense" their microenvironments (98). The τ_Δ value was estimated to be around 12 and 36 μs in membranes composed of polyunsaturated and saturated lipids, respectively, by measuring decays in bulk solutions of the phospholipids and estimating their concentrations in the membranes (100). Interestingly, Bacellar and co-workers have used the diffusion–reaction-decay properties of $^1O_2$ to investigate the O_2 distribution in the fluid/gel phases of lipid membranes (98).

The NIR emission has also been used to study $^1O_2$ in cells. There are two main protocols, one involving suspension of cells in media followed by quick measurements and another involving NIR emission devices coupled to microscopes to realize $^1O_2$ experiments in single cells. In the suspension experiments, suppression experiments (ascorbate or bovine serum albumin (BSA), for example) are used to show that the emission comes from within the cells. While in isotropic medium, the presence of ascorbate virtually suppresses all $^1O_2$ emission; in cell suspensions, τ_Δ remains almost the same in the presence or absence of ascorbate (101). Whether or not $^1O_2$ will leak out from cells, depends on many experimental parameters, such as the site of its generation and the amount of $^1O_2$ that will escape cells. Suppression experiments can be used to test the exit of $^1O_2$ from cells. By using BSA as an extra-cellular suppressor, Jiménez-Banzo and coworkers found that $^1O_2$ may or may not leak out from the cells, depending on the photosensitizer and on their intracellular location (102). Interestingly, in cells previously incubated with cold D_2O, τ_Δ was shown to depend on the type of photosensitizer, being 5 μs for a photosensitizer that localizes in mitochondria up to 33 μs for a photosensitizer that distributes in many cell compartments (101). Researchers have also studied emission properties of $^1O_2$ in suspended cells, *in vivo*, and other heterogeneous media, wherein τ_Δ is dependent on the type of photosensitizer and its location (103–108).

The intrinsic emissive properties of $^1O_2$ put extraordinary challenges for its use as an imaging probe in cell experiments. Nevertheless, successful attempts that were made should be mentioned. Observation by epifluorescence of cells loaded with photosensitizers is a suitable approach to obtain kinetic profiles of $^1O_2$ generation and decay in whole cells. This was achieved by collecting NIR light of a whole cell and directing it to a NIR-photomultiplier tube (NIR-PMT). Under these conditions, Kuimova and co-authors also observed that the τ_Δ varied with the type of photosensitizers from 30–40 μs for an aqueous soluble photosensitizer down to 4.5 μs for a membrane soluble photosensitizer, and the $^1O_2$ suppression constants by added NaN_3 were also different (109). Therefore, it is evident that τ_Δ is highly dependent on the characteristics of the photosensitizers and on their location inside cells. We can speculate that these changes in τ_Δ values are due to different suppressors present in the specific locations of the cell environments, but the molecular mechanisms are still under investigation. It is also worth mentioning the direct excitation of O_2 with a 765 nm femto-second laser, providing expected τ_Δ in sensitizer-free solvents and spatially resolved generation while triggering lethal effects in human tumor cells (43).

Electron paramagnetic resonance (EPR)

Electron paramagnetic resonance (EPR) spectroscopy, also named electron spin resonance (ESR), is a technique to study chemical species with one or more unpaired electrons (organic and inorganic free radicals, complexes with transition metal ions, etc) (110). The method is based on the excitation of electron spins and, hence, only paramagnetic species give rise to EPR signals. Since almost all biomolecules and solvents are diamagnetic, this technique shows great specificity.

Many intermediates in photosensitization reactions have unpaired electrons. However, these radicals are in general very reactive and, in consequence, possess short lifetimes in solutions. This explains that usually under steady-state conditions, the concentrations of oxygen radical species and organic radicals are very low and difficult to detect by EPR. Thus, EPR-spin trapping is an alternative analytical technique that is also used for the detection and identification of short-lived free radicals. Spin traps are compounds, susceptible to react with radical intermediates to form stable radical adducts that are detectable and fingerprintable by EPR spectroscopy (111,112). Nitrones are common reagents used as spin traps, for instance, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and α-(4-pyridyl-1-oxide) *N*-*t*-butylnitrone (PBN).

Reaction of a given spin trap with different radical species gives rise to specific radical adducts that shows, in turn, distinctive EPR spectra. Therefore, this technique that ensures the detection of organic radicals and reactive oxygen species (ROS) formed in a reaction system, provides structural information on these reactive radical species. As an example, Scheme 5 shows the formation of persistent radical adducts in the reaction of DMPO with different radical species. EPR has also been used

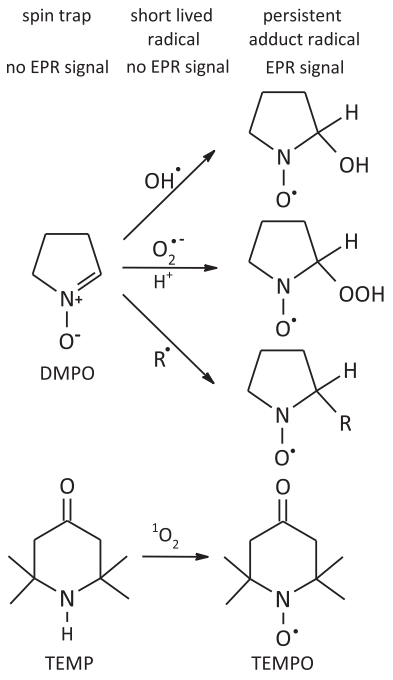
for the detection of $^1O_2$ upon trapping by 2,2,6,6-*tetra*-methylpiperidine (TEMP) to produce the free radical 2,2,6,6-*tetra*-methylpiperidine-*N*-oxyl (TEMPO), as a sterically hindered amine (Scheme 5). The total quenching rate constant (k^{Δ}_{t-TEMP}) for this reaction is ~5×10^5 M^{-1} s^{-1}. However, the most reliable detection method of $^1O_2$, particularly in biological systems is provided by the measurement of its characteristic 1270 luminescence (see above).

To study a photosensitization reaction through EPR-spin trapping the solution mixture of photosensitizers, the substrate and the spin-trap is steadily irradiated. Since the radical adducts are sufficiently long lived, the irradiation can be performed inside or outside the cavity of the EPR spectrometer. In the latter case, the irradiated solution is rapidly transferred to an EPR cell. EPR spectra are recorded at different irradiation time and increasing signals of the radicals formed are registered. Even in simple reaction systems, many radicals can be simultaneously formed (see below) and the resulting EPR spectra can be complex due to the contribution of several individual spectra.

To better detect organic radicals, such as carbon-centered radicals, for instance, those generated in type I photosensitization mechanism, O_2 can be removed from the sample before irradiation. In this way, the reactions of the spin trap with ROS are avoided, thus giving a simpler and clearer spectrum of the adduct resulting from the reaction between the spin-trap and the organic radical (113). However, even under anaerobic conditions, the recorded spectra may result from the sum of signals belonging to different organic radical species.

Scavengers

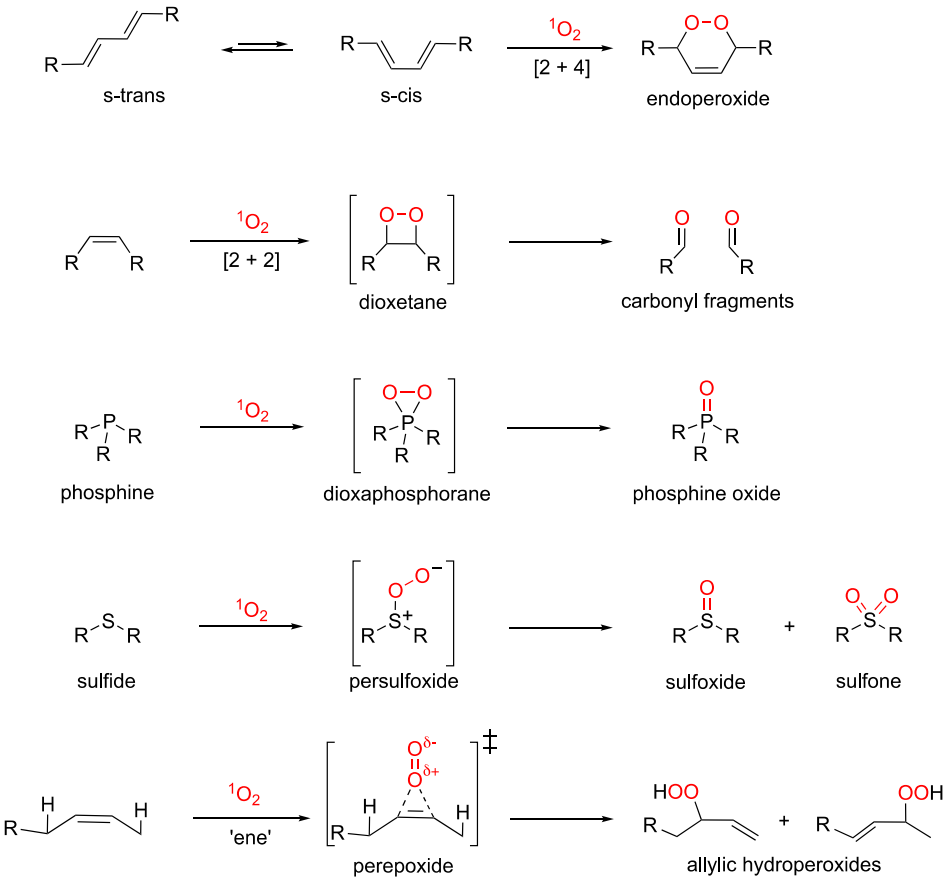
An ideal scavenger is a compound that reacts specifically with only one reactive species involved in a given photosensitization mechanism, such as the triplet excited state of the photosensitizer, an organic radical species or a ROS. Scavengers can be used in time-resolved studies, but they are more frequently used in steady-state experiments. In general, comparative experiments are performed in the presence and absence of the scavenger. If the reaction is inhibited by the scavenger, the participation of the reactive species that is quenched can be inferred. The main drawback of these experiments is the lack of selectivity. Very rarely does the scavenger react only with the target intermediate. Furthermore, the scavenger usually quenches the triplet excited state of the photosensitizer, which inhibits all the mechanisms of photosensitization (114). Therefore, for investigating the participation of a dedicated ROS, these experiments should be carried out after checking that the scavenger under the conditions used, mainly by varying the concentration, does not quench the excited states of the photosensitizer, and pooling evidence for potential competing ROS and substrate radicals. Such controls are not so easy to perform and, consequently, a reliable study with selective scavengers is often time consuming and requires considerable experimental efforts.



Scheme 5. Electron paramagnetic resonance (EPR): Formation of persistent radicals by the reaction of spin traps with short-lived radicals. DMPO: 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), TEMP: 2,2,6,6-*tetra*-methylpiperidine, TEMPO: 2,2,6,6-*tetra*-methylpiperidine-*N*-oxyl.

The interaction between the scavenger and the sought intermediate can be a physical deactivation or a chemical reaction, frequently named also as physical and chemical quenching, respectively. In the latter case, information on the selectivity or specificity may be gained by looking at the final products of the reaction. In general, the reactions of different ROS with a given scavenger lead to different products. Therefore, when a scavenger of a given reactive species prevents occurrence of a photosensitization reaction, detection of specific products is mechanistically relevant. Application of this experimental strategy is facilitated if the targeted products have been previously described in the literature and analytical methods are available for detecting the standards. In consequence, the investigation of products implies further additional experimental efforts and increased costs. In the next paragraphs, several examples of the use of scavengers are briefly described.

Selective $^1O_2$ scavengers are frequently used to reveal the participation of this ROS in the mechanism of photooxidation reactions. In that respect, charge-transfer physical quenchers such as sodium azide (NaN_3), DABCO or other suppressing amines are used under appropriate conditions. Avoid using large suppressor concentrations to avoid quenching the triplet exited state of the photosensitizer (115). β-Carotene can also be used as it is an energy-transfer physical quencher of $^1O_2$ (116). Scheme 6 shows several commonly used chemical quenchers of $^1O_2$, which include dienes to produce endoperoxides, and mono-alkenes to produce dioxetanes that cleave apart to generate carbonyl fragments. The use of chemical quenchers includes sulfides and phosphines (also seen in Scheme 6), as well as furans, 9,10-disubstituted anthracenes, *p*-nitrosodimethylaniline, and uric acid whose consumption can be followed by mass spectrometry, nuclear magnetic resonance (NMR), or UV–Vis spectrophotometry. These types of experiments are widespread, but the results can lead to wrong conclusions and have to be considered with caution. As mentioned before, the main weakness is usually the low selectivity of $^1O_2$ quenchers. Organic sulfide and phosphine traps are useful, but do not exhibit enough selectivity to differentiate between type I and type II mechanisms. This remark applies as well to thiols whose type I and type II photosensitized reactions can lead to many oxidized products, including disulfides (RSSR), thiosulfinates [RS(=O)SR], and thiosulfonates [RS(O_2)SR]. One exception concerns the selectivity of $^1O_2$ in the 'ene'



Scheme 6. $^1O_2$ scavengers of varying selectivities.

reaction in Scheme 6 that is characterized by the migration of a double bond considered as a fingerprint of the presence of $^1O_2$. Alkenes bearing an α-H that are targets for the $^1O_2$-mediated 'ene' reaction have been used to decipher relative contributions of type I and type II reactions (117,118), where the migration of a double bond monitored by NMR is useful as the analytical detection technique. In terms of NMR spectroscopy, the direct detection of intermediates by NMR can be challenging, although some low temperature NMR experiments involving organic solvent-soluble traps have been successful (114,119).

In terms of secondary $^{\bullet}$OH formed in type I reactions, D-mannitol (*e.g.* 20–100 mM), ethanol or methanol (100 mM, also up to 1 M) are good scavengers (120). Conversion of DMSO to methanesulfinic acid is characteristic of $^{\bullet}$OH (*e.g.* 100 mM). Glutathione, 2-mercaptoethanol, and dithiothreitol can be used as oxygen radical scavengers, although they are not selective. Alcohols are not good scavengers of $O_2^{\bullet-}$, their low acidity results in inefficient proton transfer as the *p*Ka of $HO_2^{\bullet}$ is ~4.8. Instead, superoxide dismutase (SOD) can be used (*e.g.* 100 μg mL^{-1}), as well as chemiluminescence methods with luminol and 6-(4-methoxyphenyl)-2-methyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one hydrochloride (MCLA) (121,122). More will be discussed on chemiluminescence methods in the next section. Catalase can similarly be used to decompose H_2O_2 (*e.g.* 100 μg mL^{-1}), and tested against denatured catalase (*e.g.* 100 μg mL^{-1}). More is described on H_2O_2 and ROS detection next, in the context of fluorescent probes.

Probes

An ideal probe is a compound that reacts specifically with only one reactive species and gives rise to a product that can be detected spectroscopically. Many probes drastically change their fluorescence quantum yield upon reaction with an intermediate, thereby increasing or decreasing their fluorescence intensity. As with the case of studies using scavengers (see above), comparative steady-state photolysis can be performed in the presence and absence of the dedicated probes. Ideally, the probe forms a product in the presence of the ROS which is spectroscopically unique. In general, the advantages and disadvantages of this methodology are similar to those described for the use of scavengers.

However, there are some differences and aspects that deserve discussion. Due to the high responsiveness of fluorescence detection, the use of probes is often more sensitive than that of scavengers with the advantage of requiring low concentrations of the probe. These features make probe experiments more suitable for complex systems, including investigations in cells. It is worth mentioning that the detection of a given intermediate by a specific probe does not constitute proof of its substantial participation in the photosensitization process; in contrast, it just means that the reactive species is present in the reaction system. The photosensitivity of luminescent probes must also be taken into account. Several luminescent probes that are UV–visible light sensitive are subject to photooxidation, which can lead to product(s) that could be the same as those formed upon reaction of

the probe with the oxidant of interest. In the next paragraphs, some relevant examples of the use of probes are briefly described.

Luminescent (chemiluminescent and fluorescent) probes. Various probes are currently used to detect ROS and reactive nitrogen species in cells. However most of them lack of specificity as a main shortcoming. The 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay that is widely applied to measure ROS since already 50 years has been shown to suffer from several drawbacks and limitations (123–127). $^{\bullet}OH$ and not $O_2^{\bullet-}$ among ROS is able to induce the release of fluorescent 2′,7′-dichlorofluorescein (DCF). Furthermore the assay is not specific of ROS since one-electron oxidants, hypohalous acids and several organic radicals including thiyl radicals ($RS^{\bullet}$) have been shown to give rise to DCF. This limitation prevents one from making any relevant conclusion on the nature of ROS that are measured. Real-time monitoring of several ROS including $O_2^{\bullet-}$ and H_2O_2 is however possible using suitable fluorescent probes (128,129). The probe Singlet oxygen sensor green (SOSG) has been shown to form an endoperoxide product that shows a typical fluorescence in the visible region and also functions as a sensitizer (130). A fluorescent NanoSOSG system of polyacrylamide nanoparticles also works well to detect $^1O_2$ in cells (131), and $^1O_2$-based chemiluminescent dioxetane cleaving systems have also been reported (132).

Detection of superoxide anion radical. Hydroethidine (HE) also known as dihydroethidium is a specific chemiluminescent probe for the detection of $O_2^{\bullet-}$ giving rise through oxidation to red 2-hydroxyethidium (2-OH-E+) as a red fluorescent product (133). However this compounds has to be separated by HPLC from other fluorescent compounds such as ethidium to allow its selective detection (134,135).

Detection of H_2O_2. Aromatic boronate-based probes react quantitatively with H_2O_2, forming a phenolic product (136). However, peroxynitrite and hypochlorite react more rapidly with boronates, forming the same product. The identity of the oxidant should therefore be confirmed using genetic and/or pharmacologic approaches, as other acidic hydroperoxides (especially peroxynitrous acid) may also contribute to the oxidation of boronates.

^{18}O, ^{17}O, ^{15}N, and ^{13}C isotopic substitution. Valuable mechanistic insight can be obtained from work with isotope tracers. For example, tracer work that uses [^{18}O]-labeled 1,4-naphthalene endoperoxides provide clean chemical sources of [^{18}O]-labeled $^{18}O_2$, which are well-suited for mass spectrometry studies (137–141). Using $^{17}O_2$ gas is also useful for tracking of reaction paths by ^{17}O NMR (142), although the NMR signals can be broad. One downside is also that $^{18}O_2$ and $^{17}O_2$ gases are expensive reagents due to their low ^{17}O and ^{18}O natural abundances to achieve high enrichments. ^{13}C and ^{15}N NMR with labeled reagents for product analysis offer an analytical tool that however requires synthesis (114,119,143). The natural abundance of ^{31}P makes it readily followed by ^{31}P NMR, although compounds such as triaryl phosphines are potent oxophiles and thus not selective for differentiating type I and type II sensitized photooxidations.

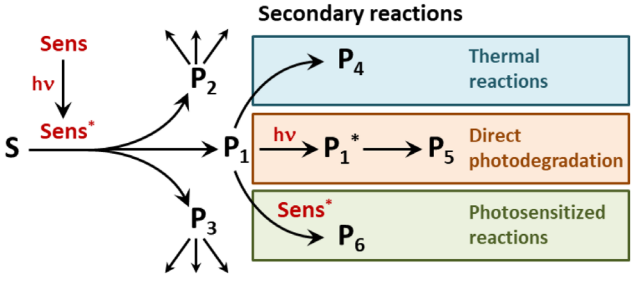
ANALYSIS OF PRODUCTS

Diversification of photosensitized damage

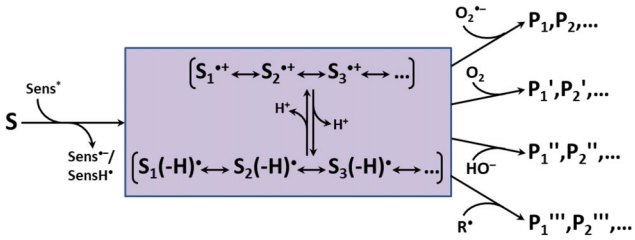
In general, different photosensitizing mechanisms operating on a given substrate lead to different primary reaction products. Knowing the main mechanism involved in the photosensitized degradation of a biomolecule helps to predict probable chemical transformation and, in the opposite direction, identification of products allows to speculate on the mechanisms. However, this simple reasoning is far from easy to implement in most cases. Even when only a single mechanism is operating, several competitive degradation pathways can occur and the relative rates of these reactions depend on the environmental conditions (Scheme 7). In addition, many primary products, such as hydroperoxides and endoperoxides, are not stable and undergo further thermal reactions leading to secondary products. Finally, both primary and secondary products can act as substrates of photosensitization reactions, can suffer photodegradation by direct absorption of radiation or can act as photosensitizers themselves. In consequence, it is clear that even in a very simple reaction system, such as an aqueous solution containing a photosensitizer and a substrate exposed to UVA radiation, many products can be generated and the content of the photosensitized solution will depend on the experimental conditions and the irradiation time.

In type I mechanisms, for instance, the radical formed by electron transfer or hydrogen abstraction frequently is more acidic than its precursor and two acid base forms can be in equilibrium. In addition, the unpaired electron is delocalized over several atoms resulting in different resonance structures. Therefore, a "free radical" is, as a matter of fact, a set of chemical structures that can participate in simultaneous pathway leading to modifications in different positions of the molecule (Scheme 8). Moreover, these structures can react with several species to yield different products. Typically, cation radicals can undergo hydration that often yields C-carbon centered radicals, reaction with O_2, by either addition or by one-electron oxidation, reaction with $O_2^{\bullet-}$, dimerize, *etc*. In addition, deprotonation is often a significant competitive reaction that leads to neutral radicals.

Analogous considerations can be made for the other mechanisms. Oxidations involving $^1O_2$ are, in general, more selective than those included in type I mechanisms. However, this ROS can react in different ways and in different sites in the same molecule. The substrate in an electronic excited state, resulting from photosensitization by TTET, can react through different pathways, depending on the experimental conditions. Even the



Scheme 7. Diversification of the photochemical damage.



Scheme 8. Competitive pathways in type I mechanism. $S_n^{\bullet+}$ and $S_n(-H)^{\bullet}$, resonance forms of the substrate radical cation and neutral radical, respectively.

formation of photoadducts can lead to a family of isomers, where the substrate and the photosensitizer are linked in different ways. A description of the countless possible chemical modifications that biomolecules can undergo due to photosensitization is beyond the aims of this review. However, to show the complexity of the issue and to orientate the reader on how to deal with the distribution of products, some relevant examples on isolated and cellular DNA will be presented in the next sections.

Products of type I and type II photosensitized degradation of isolated DNA

Detailed mechanistic information is available on the reactivity of the oxygen species and oxidation processes triggered by photodynamic effects toward isolated DNA and related model compounds in aerated aqueous solutions. Type I photosensitizers operate mostly by one-electron oxidation of nucleobases with high preference for guanine that shows the lowest ionization potential among DNA constituents (27). The resulting unstable guanine radical cation ($Gua^{\bullet+}$) is converted into final degradation products consecutively to deprotonation and nucleophilic additions. H_2O/OH^- addition to C8 of $Gua^{\bullet+}$ leads to the formation of 8-hydroxy-7,8-dihydroguanyl radical that is one-electron oxidized by O_2 to produce 8-oxo-7,8-dihydroguanine (8-oxoGua), an ubiquitous DNA oxidation product, whereas competitive one-electron reduction generates 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) (144,145). Deprotonation of $Gua^{\bullet+}$ gives rise to a highly oxidizing guanine radical ($Gua(-H)^{\bullet}$) that preferentially reacts with $O_2^{\bullet-}$ (146) mostly produced by O_2-mediated oxidation of photo-induced radical anion of the photosensitizer as part of type I mechanism. Addition of $O_2^{\bullet-}$ to radical center at C5 of the tautomeric isomer of $Gua(-H^{\bullet})$ leads through a complex multi-step pathway to the formation of 2,5-diamino-4*H*-imidazol-4-one (Iz) that is further hydrolyzed into 2,2,4-triamino-5(2*H*)-oxazolone (Oz) (147). These two guanine rearrangement products (148,149) are in addition to FapyGua (150), piperidine labile lesions in contrast to 8-oxoGua that is resistant to this treatment. It may be pointed out that $O_2^{\bullet-}$ does not exhibit any significant reactivity toward nucleobases and the 2-deoxyribose moieties (151). Singlet oxygen ($^1O_2$) that is the only ROS produced by type II photosensitization reacts exclusively with guanine among DNA components (152,153). This leads to the exclusive formation in double-stranded DNA of 8-oxoGua that is also generated by either one-electron oxidation or $^{\bullet}OH$ oxidation of guanine (144,145). Therefore the measurement of 8-oxoGua alone cannot be used of diagnostic for either type I or type II photosensitization mechanisms. This limitation necessitates the design of appropriated strategies that are further discussed below. In addition, there are a few conditions to be fulfilled in terms of selection of model compounds and conditions of photosensitized oxidation.

2′-Deoxyguanosine is a poor model system. 2′-Deoxyguanosine (dG) that is the simplest guanine DNA model compound presenting enough solubility in aqueous solution is not suitable for investigating type I and type II photosensitization mechanisms of guanine, the preferential DNA target of these reactions at least for two main reasons. Thus, evidence has been shown that highly oxidizing ($Gua(-H)^{\bullet}$) efficiently oxidizes 8-oxoGua, one of the main primary one-electron oxidation products of Gua as soon as it is formed. As a result, secondary oxidation products including spiroiminodihydantoin (Sp) and Oz that are mechanistically irrelevant when DNA is concerned are produced at the expense of 8-oxoGua whose formation rapidly plateaus at low levels (154,155). Another matter of concern is lack of specificity of chemical $^1O_2$ oxidation reactions of the guanine in dG with respect to native DNA. In both cases Diels-Alder [4 + 2] cycloaddition of $^1O_2$ across the imidazole ring of Gua gives rise to unstable 4,8-endoperoxide that undergoes linear rearrangement into 8-hydroperoxyguanine and subsequent reduction into 8-hydroxyguanine that is in dynamic equilibrium with the more stable 8-oxoGua tautomer. This pathway that is quantitative in DNA is only minor for dG since guanine 4,8-endoperoxide mainly decomposes upon loss of a water molecule into a reactive quinoid intermediate that upon subsequent hydration and rearrangement gives rise to Sp as a primary photooxidation product (156). The formation of mechanistically irrelevant Sp in either $^1O_2$ reaction or one-electron oxidation of Gua in nucleoside/nucleotide prevents the use of these simple model compounds for investigating type I and II properties of photosensitizers.

Necessity of preventing occurrence of secondary oxidation reactions of 8-oxoGua. 8-OxoGua shows a much higher susceptibility by about two orders of magnitude than parent Gua toward oxidation reactions triggered by either $^1O_2$ (157,158) or one-electron oxidation (159,160). Therefore it is a requisite even when relevant double-stranded DNA is used as the model compound to minimize the formation of secondary Gua oxidation products. This could be achieved by exposing DNA to mild oxidation conditions that led to an overall degradation of Gua moieties lower than 10%.

Measurement of oxidized nucleobases/nucleosides in isolated DNA. High performance liquid chromatography associated with accurate and sensitive electrospray ionization tandem mass spectrometry detection (HPLC-ESI-MS/MS) has been shown to be the gold standard method to quantitatively measure a large number of oxidized 2′-deoxyribonucleosides including 8-oxodG and FapydG (161). The analysis requires application of optimized condition of enzymatic digestion of oxidized DNA that leads to a quantitative release of free 2′-deoxyribonucleosides. The detection of the 2′-deoxyribonucleoside of FapyGua is more problematic since the opening of imidazole ring is accompanied by a significant increase in the weakness of the *N*-glycosidic bond of the modified nucleoside. This leads to furanose-pyranose ring isomerization and C1′-anomerization of the osidic moiety in a dynamic way that is accompanied by irreversible release of the

free base (162). This difficulty has been overcome by letting the unstable 2′-deoxyribonucleoside quantitatively converted into FapyGua that is analyzed by gas-chromatography coupled with mass spectrometry (GC–MS) once the modified base has been pre-purified by HPLC (163). A more recently alternative protocol involves direct HPLC-ESI-MS/MS analysis of released FapyGua (161). $^1O_2$ oxidation of double-stranded DNA leads to the predominant formation of 8-oxodG whereas type I photosensitized oxidation gives rise to 8-oxodG, dOz and FapyGua as the main base degradation products. The latter base degradation pattern that was observed for riboflavin, benzophenone and 2-methyl-1,4-napthoquinone, all well-documented type I photosensitizers (164) is also characteristic of type="InGeneral_Punctuation">•OH-mediated guanine degradation distribution. In addition, •OH is also capable to oxidize pyrimidine bases, however with usually a much higher efficiency than type I photosensitizers (145).

Covalently guanine-lysine cross-link represents another relevant DNA modification of type I photosensitization reaction that could be used as diagnostic of one-electron oxidation of guanine in DNA-protein complexes. The specific formation of the amino-nucleobase adducts involves covalent attachment of Ɛ-free amino group of central lysine of bound KKK tripeptide to TGT trinucleotide at guanine C8 upon riboflavin photosensitization (165).

Site specific distribution of oxidized bases in defined sequence double-stranded DNA fragments. Another relevant analytical approach has been developed for gaining mechanistic insights into type I and type II photosensitized formation in double-stranded DNA fragments. This is based on the formation of the main guanine modifications including 8-oxoGua, Oz and FapyGua at the nucleotide level. For this purpose, [^{32}P]-end labeled of double stranded DNA fragments of defined sequence that were obtained by specific restriction enzymatic cleavage of human genes were used as the oxidation DNA targets. 8-OxoGua, the exclusive $^1O_2$ oxidation product of DNA, could be revealed together with FapyGua as DNA repair glycosylase-sensitives site whereas Oz and FapyGua could be detected as alkali-labile sites upon hot piperidine treatment. Localization of enzymatic or chemical cleaved DNA sites is achieved by high resolution polyacrylamide gel electrophoresis (PAGE) analysis using the performant sequencing protocol developed by Maxam and Gilbert (166). It was found that the distribution of $^1O_2$ mediated 8-oxoGua does not show any significant sequence selectivity as observed upon photosensitization by either hematoporphyrin or lomefloxacin in agreement with the similar reactivity of guanine moieties toward $^1O_2$ oxidation in DNA fragments irrespective of the nature of vicinal bases (167–169). In contrast, the type I photosensitized formation of 8-oxoGua and piperidine-sensitive guanine lesions consisting of Oz and FapyGua is highly dependent on the nature of the flanking bases (169). Thus 5′-GG-3′ doublet and particularly 5′G exhibit a high susceptibility to $^1O_2$ oxidation that is a characteristic feature of type I photosensitizers as observed for riboflavin (167,170), methylene blue (171), nalidixic acid (168), pterins (170,172) and Nile blue (170). This was rationalized by theoretical studies in terms of decrease in the ionization potential of 5′G *versus* 3′G and 5′-localization of HOMO in stacked G doublet (173). It was also shown both experimentally and theoretically that G triplet is another preferential target for one-electron oxidation of nucleobases in native DNA with a preference for central G (174). In contrast, isolated guanines are poorly oxidized by one-electron oxidants with no base specificity.

Other analytical approaches that are based on [^{18}O]-labeling experiments have been applied for assessing contribution of type I and II photosensitization mechanisms to oxidation reactions of DNA. Thus riboflavin-mediated sensitization of calf thymus DNA to UVA radiation in [^{18}O]-labeled aqueous solution led to the formation of [^{18}O]-enriched 8-oxodG as a relevant indicator of type I photosensitization mechanism through specific hydration of the guanine radical cation (175). Monitoring the formation of monoatomic labeled 8-oxodG arising from [^{18}O]-$^1O_2$ oxidation exposure (176) could be used as a relevant indicator of type II photosensitization reaction in aerated aqueous solution of DNA. Enhancement of photosensitized formation of 8-oxodG in D_2O that is explained by a significant increase of $^1O_2$ lifetime in deuterated solvent (61) constitutes an alternative and complementary way to assess the implication of type II mechanism as shown for hematoporphyrin (141).

Cellular DNA

Abundant information is available on the chemical reactions triggered by both type I and type II photosensitization reactions that result in the predominant oxidation of the guanine base in isolated DNA (144,145). Interestingly the aqueous solution model system that has been mostly used to investigate oxidizing reactions of the guanine moiety mediated by $^1O_2$, one-electron oxidants and (•OH) has been shown to be biologically relevant in terms of reactivity and formation of final degradation products in cellular DNA (34,145,177). Therefore, measurement of selected biomarkers such as 8-oxodG and FapyGua in cellular DNA could be of mechanistic value. However, this requires application of accurate and sensitive methods since the levels of oxidatively generated DNA modifications are at best in the range of a few modifications per 10^6 nucleosides. Another difficulty that further complicates investigation of photosensitized reactions of cellular DNA is the possible implication of •OH–mediated degradation processes triggered by Fenton reactions as part of biochemical oxidative stress response to UV radiation (178,179).

Methods of measurement of photosensitized DNA damage. HPLC-ESI-MS/MS is the method of choice to measure 8-oxodG and FapyGua the two main oxidatively generated guanine modifications in cellular DNA (161). However occurrence of spurious oxidation of overwhelming normal nucleosides during DNA extraction and subsequent work-up that cannot be totally prevented (145,161,180) could represent a limitation when mild conditions of photosensitization are concerned. A more sensitive but less specific alternative to detect oxidatively base lesions is to apply highly sensitive modified alkaline comet assay that in contrast to HPLC methods is exempt of artifactual oxidation occurrence (181,182). Pre-incubation of released DNA by suitable DNA repair glycosylases allow highly sensitive detection and quantitation of two main classes of modified bases. Thus digestion of DNA with either formamidopyrimidine DNA *N*-glycosylase or mammalian 8-oxoguanine DNA *N*-glycosylase allows measurement of modified purine bases consisting predominantly of 8-oxoGua and FapyGua. In addition, the levels of 5,6-saturated pyrimidine bases could be assessed upon DNA incubation with bacterial endonuclease III (endo III). Application of the

standard protocol that omits DNA repair incubation provides information on the generation of oligonucleotide strand breaks including direct DNA nicks and alkali-labile lesions.

UVA photosensitization of DNA in human cells. The implication of oxidation reactions with a preponderant role played by $^1O_2$ in the deleterious effects of UVA on human skin cells was demonstrated by the initial contributions of RM Tyrell and collaborators (183,184). Evidence was subsequently provided that UVA irradiation triggered the predominant formation of formamidopyrimidine (Fpg)-sensitive sites that were revealed by alkaline elution technique (185,186). More specifically 8-oxodG an ubiquitous DNA oxidation was measured by HPLC-ECD and HPLC- MS/MS (for an early review, see Ref. (187) and herein references). It was also shown that 8-oxodGuo, the main oxidation product, is generated as relatively minor lesions with respect to predominant cyclobutane pyrimidine dimers (CPDs) in human fibroblasts, keratinocytes (188) and skin explants (189) upon UVA irradiation. While predominant CPDs are formed by direct UVA excitation of pyrimidine bases, 8-oxodG and other oxidatively minor oxidatively generated DNA modifications arise from implication of still hypothetical endogenous photosensitizers which nature is likely to differ according to cells (179). Mechanistic insights into UVA-sensitized oxidation DNA degradation pathways of human monocytes TPH1 were gained from the measurement of 8-oxodG and FapyGua, two relevant oxidatively produced guanine modifications by HPLC-ECD and HPLC/GC/MS respectively. As striking results it was found that 8-oxodG is predominantly generated whereas no significant increase in the level of FapyGua was observed with respect to control, thus strongly suggesting that type II mechanism is mostly involved in the photosensitization reactions of monocyte DNA. Further information was provided by the measurements of three main classes of DNA oxidation damage using both the basic and enzymatic versions of the alkaline comet assay. It was found that Fpg-sensitive sites consisting of guanine modifications, likely 8-oxodG, are predominantly produced whereas the formation of relatively minor endo III-sensitive sites and direct DNA strand breaks/alkali-labile sites is mostly accounted for $^{\bullet}OH$ mediated degradation reactions (Table 2). This is supported by the comparison of the distribution of the three classes of DNA UVA modifications with that produced by gamma rays (Table 2) that predominantly generate $^{\bullet}OH$ as the reactive species whereas ionization is a minor contributor. Interestingly the relative yield of DNA strand breaks with respect to pyrimidine base modifications (about 3) is similar in DNA exposed to either UVA radiation or gamma rays. However in agreement with the mild oxidizing feature of $^1O_2$ that exclusivity generated 8-oxodG, the relative yield of Fpg-sensitive sites compared to DNA stand breaks is about 6-fold higher upon UVA irradiation than exposure to gamma rays. It may be concluded that oxidation reactions triggered by UVA on monocytes are predominantly accounted for by photosensitizers operating through type II photosensitization mechanism together with a minor contribution of $^{\bullet}OH$ issued from Fenton type reactions as part of biochemical responses to UVA irradiation. Information is also available on the respective levels of 8-oxodG, Fpg-sensitive sites and strand breaks in the DNA of human keratinocytes and melanocytes growth from the same donor (190). The UVA-induced formation of 8-oxodG and Fpg-sites is about two-fold higher in melanocytes than in keratinocytes in agreement with earlier observations (191). On the other hand the levels of DNA strand breaks that include alkali-labile sites are similar in both type cells (190). The relative increase in the levels of guanine modifications by comparison with DNA breaks in melanocytes than in keratinocytes is suggestive of relative higher contribution of $^1O_2$ with respect to Fenton reaction occurrence. Evidence has been provided from spectroscopic studies (192) and visible light irradiation of biological samples (92,193) that melanin is able to generate $^1O_2$ and also $O_2^{\bullet-}$, as a likely precursor of $^{\bullet}OH$ in cells. There is a strong need of assessing the photodynamic features of a wide variety of photosensitizers on cellular DNA that could be done by measuring both specific lesions and broader classes of damage. This concerns in particular phototherapeutic thiazine dyes with emphasis on methylene blue that bind to DNA and induce in addition to 8-oxodG other unknown modifications (194,195), likely as the result of involvement of both types I and type II photosensitization mechanisms (196–198).

Table 2. Relative yield of UVA radiation and gamma ray induced classes of DNA damage*.

Classes of oxidatively generated DNA damage	UVA radiation	Gamma rays
Fpg-sensitive sites (purine modifications)	61%	21%
Endo III-sensitive sites (pyrimidine base lesions)	10%	23%
DNA strand breaks (direct and alkali-labile sites)	29%	56%

*Measured by basal and enzymatic versions of the alkaline comet assay (181).

DO'S AND DON'TS FOR GOOD PRACTICES

Scheme 1 shows the types of photosensitization reaction involving biological targets. There are four quadrants, where the above text provided practical aspects on how to study them. They include oxygen-dependent (type I and type II processes) and oxygen-*in*dependent sensitization (TTET and photoadduct formation). The first recommendation clearly is to consider the four mechanisms and do not restrict the analysis to the more widely broadcast and known type I and type II mechanisms.

Insight into these four mechanisms is provided and how to assess their relative contributions and being critical in the analysis. Isolated system *vs*. cellular system: There are pluses and minuses to each since the number of variables in the former is lower but in a contrived setting, while in the latter, a larger number of variables complicates a "high-resolution" mechanistic analysis. For example, in cellular systems, there are challenges from competing (*e.g.* $O_2^{\bullet-}$, HO•, NO•, $ONOO^-$, OCl^-, H_2O_2, Fenton, and other oxidation and autoxidation reactions) that also arise by normal enzymatic reactions. In short, one must be very careful in applying conclusions obtained from simple and controlled systems to cells. There are virtues in putting together different possibilities that associate physical, chemical, and biological aspects. Therefore use of various sources of information from isolated and cellular systems could be useful.

In this survey we have described the use of different techniques and different experimental strategies, emphasizing the pros and cons of each one. It is clear that no reliable conclusions

can be made and no elucidation of mechanisms is possible using a single technique. To sum up, there are benefits that can be expected by using different methodologies and strategies.

CONCLUSIONS

This is a follow-up of recent surveys aimed at reviewing mechanistic aspects of photosensitized degradation of key biomolecules (nucleic acids, unsaturated lipids, proteins) that have included a slightly revisited version of type I and type II photosensitization pathways. Emphasis is placed in the present article on the evaluation of the potential and limitation of available experimental approaches for providing mechanistic insights in photosensitized reactions. These include time-resolved and steady-state spectroscopic methods that may be complemented by the delineation of the modifying effects of quenchers and scavengers. In addition, relevant information may be gained from the characterization of dedicated final degradation products of biomolecules, information that can be used in cells as relevant and sensitive biomarker(s) of representative photosensitized mechanisms. We hope our work will establish good standard practices, with clear hints on how to succeed in the study of the photosensitized oxidation reactions.

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REFERENCES

1. Cadet, J. and T. Douki (2011) Oxidatively generated damage to DNA by UVA radiation in cells and human skin. *J. Invest. Dermatol.* **131**, 1005–1007.
2. Pattison, D. I., A. S. Rahmanto and M. J. Davies (2012) Photooxidation of proteins. *Photochem. Photobiol. Sci.* **11**, 38–53.
3. Bacellar, I. O. L. and M. S. Baptista (2019) Mechanisms of photosensitized lipid oxidation and membrane permeabilization. *ACS Omega* **4**, 21636–21646.
4. El Ghissassi, F., R. Baan, K. Straif, Y. Grosse, B. Secretan, V. Bouvard, L. Benbrahim-Tallaa, N. Guha, C. Freeman, L. Galichet and V. Cogliano (2009) A review of human carcinogens—Part D: Radiation. *Lancet Oncol.* **10**, 751–752.
5. Buckel, T. B. H., A. M. Goldstein, M. C. Fraser, B. Rogers and M. A. Tucker (2006) Recent tanning bed usea risk factor for melanoma. *Arch. Dermatol.* **142**, 485–488.
6. Ting, W., K. Schultz, N. N. Cac, M. Peterson and H. W. Walling (2007) Tanning bed exposure increases the risk of malignant melanoma. *Int. J. Dermatol.* **46**, 1253–1257.
7. Moan, J., A. C. Porojnicu and A. Dahiback (2008) Ultraviolet radiation and malignant melanoma. *Adv. Exp. Med. Biol.* **624**, 104–116.
8. Manjón, F., L. Villén, D. García-Fresnadillo and G. Orellana (2008) On the factors influencing the performance of solar reactors for water disinfection with photosensitized singlet oxygen. *Environ. Sci. Technol.* **42**, 301–307.
9. Agazzi, M. L., M. B. Ballatore, A. M. Durantini, E. N. Durantini and A. C. Tomé (2019) BODIPYs in antitumoral and antimicrobial photodynamic therapy: An integrating review. *J. Photochem. Photobiol. C* **40**, 21–48.
10. Wainwright, M., T. Maisch, S. Nonell, K. Plaetzer, A. Almeida, G. P. Tegos and M. R. Hamblin (2017) Photoantimicrobials—Are we afraid of the light? *Lancet Infect. Dis.* **17**, 49–55.
11. Loeb, S., R. Hofmann and J.-H. Kim (2016) Beyond the pipeline: Assessing the efficiency limits of advanced technologies for solar water disinfection. *Environ. Sci. Technol. Lett.* **3**, 73–80.
12. Castano, A. P., P. Mroz and M. R. Hamblin (2006) Photodynamic therapy and anti-tumour immunity. *Nat. Rev. Cancer* **6**, 535–545.
13. Casas, A. (2020) Clinical uses of 5-aminolaevulinic acid in photodynamic treatment and photodetection of cancer: A review. *Cancer Lett.* **490**, 165–173.
14. Abrahamse, H. and M. R. Hamblin (2016) New photosensitizers for photodynamic therapy. *Biochem. J.* **473**, 347–364.
15. Wondrak, G. T., M. K. Jacobson and E. L. Jacobson (2006) Endogenous UVA-photosensitizers: Mediators of skin photodamage and novel targets for skin photoprotection. *Photochem. Photobiol. Sci.* **5**, 215–237.
16. Sherin, P. S., N. P. Gritsan and Y. P. Tsentalovich (2009) Experimental and quantum chemical study of photochemical properties of 4-hydroxyquinoline. *Photochem. Photobiol. Sci.* **8**, 1550–1557.
17. Sormacheva, E. D., P. S. Sherin and Y. P. Tsentalovich (2017) Dimerization and oxidation of tryptophan in UV-A photolysis sensitized by kynurenic acid. *Free Radic. Biol. Med.* **113**, 372–384.
18. Savina, E. D., Y. P. Tsentalovich and P. S. Sherin (2020) UV-A induced damage to lysozyme via type I photochemical reactions sensitized by kynurenic acid. *Free Radic. Biol. Med.* **152**, 482–493.
19. Floyd, R. A., J. E. Schneider Jr. and D. P. Dittmer (2004) Methylene blue photoinactivation of RNA viruses. *Antivir. Res.* **61**, 141–151.
20. Pathak, M. A. and D. M. Krämer (1969) Photosensitization of skin in vivo by furocoumarins (psoralens). *Biochim. Biophys. Acta* **195**, 197–206.
21. Pathak, M. A. and T. B. Fitzpatrick (1992) The evolution of photochemotherapy with psoralens and UVA (PUVA): 2000 BC to 1992 AD. *J. Photochem. Photobiol. B* **14**, 3–22.
22. Westberg, M., M. Etzerodt and P. R. Ogilby (2019) Rational design of genetically encoded singlet oxygen photosensitizing proteins. *Curr. Opin. Struct. Biol.* **57**, 56–62.
23. Endres, S., M. Wingen, J. Torra, R. Ruiz-Gonzalez, T. Pollen, G. Bosio, N. L. Bitzenhofer, F. Hilgers, T. Gensch, S. Nonell, K. E. Jaeger and T. Drepper (2018) An optogenetic toolbox of LOV-based photosensitizers for light-driven killing of bacteria. *Sci. Rep.* **8**, 1–14.
24. Ruiz-Gonzalez, R., A. L. Cortajarena, S. H. Mejias, M. Agut, S. Nonell and C. Flors (2013) Singlet oxygen generation by the genetically encoded tag miniSOG. *J. Am. Chem. Soc.* **135**, 9564–9567.
25. Pimenta, F. M., R. L. Jensen, T. Breitenbach, M. Etzerodt and P. R. Ogilby (2013) Oxygen-dependent photochemistry and photophysics of "MiniSOG," a protein-encased flavin. *Photochem. Photobiol.* **89**, 1116–1126.
26. Baptista, M. S., J. Cadet, A. Greer and A. H. Thomas (2021) Photosensitization reactions of biomolecules: Definition, targets and mechanisms. *Photochem. Photobiol.* **97**, 1456–1483.
27. Baptista, M. S., J. Cadet, P. Di Mascio, A. A. Ghogare, A. Greer, M. R. Hamblin, C. Lorente, S. C. Nunez, M. S. Ribeiro, A. H. Thomas, M. Vignoni and T. M. Yoshimura (2017) Type I and II photosensitized oxidation reactions: Guidelines and mechanistic pathway. *Photochem. Photobiol.* **93**, 912–919.
28. Foote, C. S. (1991) Definition of type I and type II photosensitized oxidation. *Photochem. Photobiol.* **54**, 659.
29. Jensen, P.-G., J. Arnbjerg, L. P. Tolbod, R. Toftegaard and P. R. Ogilby (2009) Influence of an intermolecular charge-transfer state on excited-state relaxation dynamics: Solvent effect on the methylnaphthalene-oxygen system and its significance for singlet oxygen. *J. Phys. Chem. A* **113**, 9965–9973.
30. Arnbjerg, J., M. Johnsen, C. B. Nielsen, M. Jorgensen and P. R. Ogilby (2007) Effect of sensitizer protonation on singlet oxygen production in aqueous and nonaqueous media. *J. Phys. Chem. A* **111**, 4573–4583.
31. Schmidt, R. (2005) The balance between charge transfer and non-charge transfer pathways in the sensitization of singlet oxygen by $\pi\pi^*$ triplet states. *Photochem. Photobiol. Sci.* **4**, 481–486.
32. Schweitzer, C., Z. Mehrdad, F. Shafii and R. Schmidt (2001) Charge transfer induced quenching of triplet sensitizers by ground state oxygen and of singlet oxygen by ground state sensitizers: A common deactivation channel. *Phys. Chem. Chem. Phys.* **3**, 3095–3101.

33. Baklanov, A. V., A. S. Bogomolov, A. P. Pyryaeva, G. A. Bogdanchikov, S. A. Kochubei, Z. Farooq and D. H. Parker (2015) Singlet oxygen photogeneration from X-O2 van der Waals complexes: Double spin-flip vs. charge-transfer mechanism. *Phys. Chem. Chem. Phys.* **17**, 28565–28573.
34. Di Mascio, P., G. R. Martinez, S. Miyamoto, G. E. Ronsein, M. H. G. Medeiros and J. Cadet (2019) Singlet molecular oxygen reactions with nucleic acids, lipids, and proteins. *Chem. Rev.* **119**, 2043–2086.
35. Foote, C. S. (1968) Mechanisms of photosensitized oxidation. *Science* **162**, 963–970.
36. Redmond, R. W. and I. E. Kochevar (2006) Spatially resolved cellular responses to singlet oxygen. *Photochem. Photobiol.* **82**, 1178–1186.
37. Greer, A. (2006) Christopher Foote's discovery of the role of singlet oxygen [$^1O_2$ ($^1\Delta_g$)] in photosensitized oxidation reactions. *Acc. Chem. Res.* **39**, 797–804.
38. Ghogare, A. A. and A. Greer (2016) Using singlet oxygen to synthesize natural products and drugs. *Chem. Rev.* **116**, 9994–10034.
39. Ogilby, P. R. (2010) Singlet oxygen: There is indeed something new under the sun. *Chem. Soc. Rev.* **39**, 3181–3209.
40. Levine, I. N. (2000) *Quantum Chemistry*, 5th ed., pp. 400–406. Prentice Hall, Upper Saddle River, NJ.
41. Roslaniec, M. C. and E. M. Sanford (2011) Benzoylation of ergosterol through nucleophilic acyl substitution and subsequent formation of ergosterol benzoate endoperoxide by reaction with singlet oxygen generated by photosensitization. *J. Chem. Ed.* **88**, 229–231.
42. Tonon, C. C., S. Ashraf, J. Q. Alburquerque, A. Nara de Sousa Rastelli, T. Hasan, A. M. Lyons and A. Greer (2021) Antimicrobial photodynamic inactivation using topical and superhydrophobic sensitizer techniques: A perspective from diffusion in biofilms. *Photochem. Photobiol.* **97**, 1266–1277.
43. Bregnhøj, M., A. Blázquez-Castro, M. Westberg, T. Breitenbach and P. R. Ogilby (2015) Direct 765 nm optical excitation of molecular oxygen in solution and in single mammalian cells. *J. Phys. Chem. B* **119**, 5422–5429.
44. Blázquez–Castro, A., T. Breitenbach and P. R. Ogilby (2018) Cell cycle modulation through subcellular spatially resolved production of singlet oxygen via direct 765 nm irradiation: Manipulating the onset of mitosis. *Photochem. Photobiol. Sci.* **17**, 1310–1318.
45. Qian, H., M. E. Cousins, E. H. Horak, A. Wakefield, M. D. Liptak and I. Aprahamian (2017) Suppression of Kasha's rule as a mechanism for fluorescent molecular rotors and aggregation-induced emission. *Nat. Chem.* **9**, 83–87.
46. Rehm, D. and A. Weller (1970) Kinetics of fluorescence quenching by electron and H-atom transfer. *Isr. J. Chem.* **8**, 259–271.
47. Eberson, L. (1982) Electron transfer reactions in organic chemistry. *Adv. Phys. Org. Chem.* **18**, 79–185.
48. Franco, C. and J. Olmsted III (1990) Photochemical determination of the solubility of oxygen in various media. *Talanta* **37**, 905–909.
49. Butler, I. B., M. A. A. Schoonen and D. T. Rickard (1994) Removal of dissolved oxygen from water: A comparison of four common techniques. *Talanta* **41**, 211–215.
50. Sato, T., Y. Hamada, M. Sumikawa, S. Araki and H. Yamamoto (2014) Solubility of oxygen in organic solvents and calculation of the Hansen solubility parameters of oxygen. *Ind. Eng. Chem. Res.* **53**, 19331–19337.
51. Vencel, T., J. Donovalová, A. Gáplovský, T. Kimura and Š. Toma (2005) Oxygen exclusion from the organic solvents using ultrasound and comparison with other common techniques used in photochemical experiments. *Chem. Pap.* **59**, 271–274.
52. O'Donnell, R. M., T. A. Grusenmeyer, D. J. Stewart, T. R. Ensley, W. M. Shensky III, J. E. Haley and J. Shi (2017) Photodriven oxygen removal via chromophore-mediated singlet oxygen sensitization and chemical capture. *Inorg. Chem.* **56**, 9273–9280.
53. Sasso, M.,. G., F. H. Quina and E. J. H. Bechara (1986) Ruthenium (II) tris(bipyridyl) ion as a luminescent probe for oxygen uptake. *Anal. Biochem.* **156**, 239–243.
54. Junqueira, H. C., D. Severino, L. G. Dias, M. S. Gugliotti and M. S. Baptista (2002) Modulation of methylene blue photochemical properties based on adsorption at aqueous micelle interfaces. *Phys. Chem. Chem. Phys.* **4**, 2320–2328.
55. Tasso, T. T., J. C. Schlothauer, H. C. Junqueira, T. A. Matias, K. Araki, É. Liandra-Salvador, F. C. T. Antonio, P. Homem-de-Mello and M. S. Baptista (2019) Photobleaching efficiency parallels the enhancement of membrane damage for porphyrazine photosensitizers. *J. Am. Chem. Soc.* **141**, 15547–15556.
56. Bacellar, I. O. L., M. C. Oliveira, L. S. Dantas, E. B. Costa, H. C. Junqueira, W. K. Martins, A. M. Durantini, G. Cosa, P. Di Mascio, M. Wainwright, R. Miotto, R. M. Cordeiro, S. Miyamoto and M. S. Baptista (2018) Photosensitized membrane permeabilization requires contact-dependent reactions between photosensitizer and lipids. *J. Am. Chem. Soc.* **140**, 9606–9615.
57. Pushalkar, S., G. Ghosh, Q. Xu, Y. Liu, A. A. Ghogare, C. Atem, A. Greer, D. Saxena and A. M. Lyons (2018) Superhydrophobic photosensitizers: Airborne $^1O_2$ killing of an *in vitro* oral biofilm at the plastron interface. *ACS Appl. Mater. Interfaces* **10**, 25819–25829.
58. Al Tameemi1, W., T. P. Dale, R. M. K. Al-Jumaily and N. R. Forsyth (2019) Hypoxia-modified cancer cell metabolism. *Front. Cell Dev. Biol.* **7**, 4.
59. Tardivo, J. P., M. S. Baptista, J. A. Correa, F. Adami and M. A. S. Pinhal (2015) Development of the Tardivo algorithm to predict amputation risk of diabetic foot. *PLoS One* **10**, e0135707.
60. Lameijer, L. N., D. Ernst, S. L. Hopkins, M. S. Meijer, S. H. C. Askes, S. E. Le Dévédec and S. Bonnet (2017) A red-light-activated ruthenium-caged NAMPT inhibitor remains phototoxic in hypoxic cancer cells. *Angew. Chem. Int. Ed.* **129**, 11707–11711.
61. Ogilby, P. R. and C. S. Foote (1983) Chemistry of singlet oxygen. 42. Effect of solvent, solvent isotopic substitution, and temperature on the lifetime of singlet molecular oxygen ($^1\Delta_g$). *J. Am. Chem. Soc.* **105**, 3423–3430.
62. Schweitzer, C. and R. Schmidt (2003) Physical mechanisms of generation and deactivation of singlet oxygen. *Chem. Rev.* **103**, 1685–1758.
63. Scurlock, R. D., S. Nonell, S. E. Braslavsky and P. R. Ogilby (1995) Effect of solvent on the radiative decay of singlet molecular oxygen ($a^1\Delta_g$). *J. Phys. Chem.* **99**, 3521–3526.
64. Martinez, L. A., C. G. Martínez, B. B. Klopotek, J. Lang, A. Neuner, A. M. Braun and E. Oliveros (2000) Nonradiative and radiative deactivation of singlet molecular oxygen ($O_2(a^1\Delta_g)$) in micellar media and microemulsions. *J. Photochem. Photobiol. B* **58**, 94–107.
65. Petroselli, G., M. L. Dántola, F. M. Cabrerizo, A. L. Capparelli, C. Lorente, E. Oliveros and A. H. Thomas (2008) Oxidation of 2′-deoxyguanosine 5′-monophosphate photoinduced by pterin: Type I versus type II mechanism. *J. Am. Chem. Soc.* **130**, 3001–3011.
66. Scaiano, J. C. (2003) Nanosecond laser flash photolysis: A tool for physical organic chemistry. In *Reactive Intermediate Chemistry*, Vol. **18** (Edited by R. A. Moss, M. S. Platz and M. Jones Jr.), pp. 847–871. John Wiley & Sons, New Jersey.
67. Barra, M., C. Bohne and J. C. Scaiano (1990) Effect of cyclodextrin complexation on the photochemistry of xanthone. Absolute measurement of the kinetics for triplet-state exit. *J. Am. Chem. Soc.* **112**, 8075–8079.
68. Serrano, M. P., C. Lorente, C. D. Borsarelli and A. H. Thomas (2015) Unraveling the degradation mechanism of purine nucleotides photosensitized by pterins: The role of charge-transfer steps. *ChemPhysChem* **16**, 2244–2252.
69. Ramel, F., S. Birtic, S. Cuine´, C. Triantaphylidès, J.-L. Ravanat and M. Havaux (2012) Chemical quenching of singlet oxygen by carotenoids in plants. *Plant Physiol.* **158**, 1267–1278.
70. Steenken, S. (1966) One-electron redox reactions between radicals and organic molecules. An addition/elimination (inner-sphere) path [1]. In Mattay, J. (Ed.), *Electron Transfer II. Topics in Current Chemistry*, Vol. **177**, pp. 126–145. Springer, Berlin, Heidelberg.
71. Gerstein, M. and C. Chothia (1996) Packing at the protein-water interface. *Proc. Natl. Acad. Sci. USA* **93**, 10167–10172.
72. Baptista, M. S. and G. L. Indig (1998) Effect of BSA binding on photophysical and photochemical properties of triarylmethane dyes. *J. Phys. Chem. B* **102**, 4678–4688.
73. Ashur, I., R. Goldschmidt, I. Pinkas, Y. Salomon, G. Szewczyk, T. Sarna and A. Scherz (2009) Photocatalytic generation of oxygen radicals by the water-soluble bacteriochlorophyll derivative WST11,

noncovalently bound to serum albumin. *J. Phys. Chem. A* **113**, 8027–8037.
74. Wilkinson, F., D. J. McGarvey and A. F. Olea (1994) Excited triplet state interactions with molecular oxygen: Influence of charge transfer on the bimolecular quenching rate constants and the yields of singlet oxygen [$O_2(^1\Delta_g)$] for substituted naphthalenes in various solvents. *J. Phys. Chem.* **98**, 3762–3769.
75. Lee, J. I., M. Sato, K. Ushida and J. Mochida (2011) Measurement of diffusion in articular cartilage using fluorescence correlation spectroscopy. *BMC Biotechnol.* **11**, 19.
76. Vignoni, M., M. N. Urrutia, H. C. Junqueria, A. Greer, A. Reis, M. S. Baptista, R. Itri and A. H. Thomas (2018) Photo-oxidation of unilamellar vesicles by a lipophilic pterin: Deciphering biomembrane photodamage. *Langmuir* **34**, 15578–15586.
77. Zheng, D., M. Tao, L. Yu, X. Liu, A. Xia and J. Wang (2021) Ultrafast photoinduced electron transfer in a photosensitizer protein. *CCS Chem.* **3**, 1580–1586.
78. Purugganan, M. D., C. V. Kumar, N. J. Turroand and J. K. Barton (1988) Accelerated electron transfer between metal complexes mediated by DNA. *Science* **241**, 1645–1649.
79. Janda, T., G. Szalai, N. Papp, M. Pál and E. Páldi (2004) Effects of freezing on thermoluminescence in various plant species. *Photochem. Photobiol.* **80**, 525–530.
80. Matsuzaki, H., T. N. Murakami, N. Masaki, A. Furube, M. Kimura and S. Mori (2014) Dye aggregation effect on interfacial electron-transfer dynamics in zinc phthalocyanine-sensitized solar cells. *J. Phys. Chem. C* **118**, 17205–17212.
81. Khan, A. U. and M. Kasha (1979) Direct spectroscopic observation of singlet oxygen emission at 1268 nm excited by sensitizing dyes of biological interest in liquid solution. *Proc. Natl. Acad. Sci. USA* **76**, 6047–6049.
82. Kasha, M. (1985) Singlet oxygen electronic structure. In *Singlet O_2*, Vol. **1** (Edited by A. Frimer), pp. 1–12. CRC Press, Boca Raton, FL.
83. Jiménez-Banzo, A., X. Ragàs, P. Kapusta and S. Nonell (2008) Time-resolved methods in biophysics. 7. Photon counting vs. analog time-resolved singlet oxygen phosphorescence detection. *Photochem. Photobiol. Sci.* **7**, 1003–1010.
84. Gorman, A. A. and M. A. J. Rodgers (1992) Current perspectives of singlet oxygen detection in biological environments. *J. Photochem. Photobiol. B* **14**, 159–176.
85. Hurst, J. R., J. D. McDonald and G. B. Schuster (1982) Lifetime of singlet oxygen in solution directly determined by laser spectroscopy. *J. Am. Chem. Soc.* **104**, 2065–2067.
86. Wilkinson, F., W. P. Helman and A. B. Ross (1995) Rate constants for the decay and reactions of the lowest electronically excited singlet state of molecular oxygen in solution. An expanded and revised compilation. *J. Phys. Chem. Ref. Data Monogr.* **24**, 663–677.
87. Mikkel Bregnhøj, M., M. Westberg, B. F. Minaev and P. R. Ogilby (2017) Singlet oxygen photophysics in liquid solvents: Converging on a unified picture. *Acc. Chem. Res.* **50**, 1920–1927.
88. Bregnhøj, M., M. Westberg, F. Jensen and P. R. Ogilby (2016) Solvent-dependent singlet oxygen lifetimes: Temperature effects implicate tunneling and charge-transfer interactions. *Phys. Chem. Chem. Phys.* **18**, 22946–22961.
89. Uchoa, A. F., P. P. Knox, R. Turchielle, N. K. Seifullina and M. S. Baptista (2008) Singlet oxygen generation in the reaction centers of Rhodobacter sphaeroides. *Eur. Biophys. J.* **37**, 843–850.
90. Darmanyan, A. P., D. D. Gregory, Y. Guo, W. S. Jenks, L. Burel, D. Eloy and P. Jardon (1998) Quenching of singlet oxygen by oxygen- and sulfur-centered radicals: Evidence for energy transfer to peroxyl radicals in solution. *J. Am. Chem. Soc.* **120**, 396–403.
91. Thorning, F., P. Henke and P. R. Ogilby (2022) Perturbed and activated decay: The lifetime of singlet oxygen in liquid organic solvents. *J. Am. Chem. Soc.* **144**, 10902–10911.
92. Chiarelli-Neto, O., C. Pavani, A. S. Ferreira, A. F. Uchoa, D. Severino and M. S. Baptista (2011) Generation and suppression of singlet oxygen in hair by photosensitization of melanin. *Free Radic. Biol. Med.* **51**, 1195–1202.
93. Lissi, E. A., M. V. Encinas, E. Lemp and M. A. Rubio (1993) Singlet oxygen $O_2(^1\Delta_g)$ bimolecular processes. Solvent and compartmentalization effects. *Chem. Rev.* **93**, 699–723.
94. Sokolov, V. S., O. V. Batishchev, S. A. Akimov, T. R. Galimzyanov, A. N. Konstantinova, E. Malingriaux, Y. G. Gorbunova, D. G. Knyazev and P. Pohl (2018) Residence time of singlet oxygen in membranes. *Sci. Rep.* **8**, 14000.
95. Gorman, A. A., G. Lovering and M. A. J. Rodgers (1976) The photosensitized formation and reaction of singlet oxygen, $O_2(^1\Delta_g)$, in aqueous micellar systems. *Photochem. Photobiol.* **23**, 399–403.
96. Lee, P. C. and M. A. J. Rodgers (1983) Singlet molecular oxygen in micellar systems. 1. Distribution equllibria between hydrophobic and hydrophilic compartments. *J. Phys. Chem.* **87**, 4894–4898.
97. Zebger, I., L. Poulsen, Z. Gao, L. K. Andersen and P. R. Ogilby (2003) Singlet oxygen images of heterogeneous samples: Examining the effect of singlet oxygen diffusion across the interfacial boundary in phase-separated liquids and polymers. *Langmuir* **19**, 8927–8933.
98. Bacellar, I. O. L., R. M. Cordeiro, P. Mahling, M. S. Baptista, B. Röder and S. Hackbarth (2019) Oxygen distribution in the fluid/gel phases of lipid membranes. *Biochim. Biophys. Acta Biomembr.* **1861**, 879–886.
99. Hackbarth, S. and B. Röder (2015) Singlet oxygen luminescence kinetics in a heterogeneous environment – Identification of the photosensitizer localization in small unilamellar vesicles. *Photochem. Photobiol. Sci.* **14**, 329–334.
100. Ehrenberg, B., J. L. Anderson and C. S. Foote (1998) Kinetics and yield of singlet oxygen photosensitized by hypericin in organic and biological media. *Photochem. Photobiol.* **68**, 135–140.
101. Oliveira, C. S., R. Turchiello, A. J. Kowaltowski, G. L. Indig and M. S. Baptista (2011) Major determinants of photoinduced cell death: Subcellular localization versus photosensitization efficiency. *Free Radic. Biol. Med.* **51**, 824–833.
102. Jiménez-Banzo, A., M. L. Sagristà, M. Mora and S. Nonell (2008) Kinetics of singlet oxygen photosensitization in human skin fibroblasts. *Free Radic. Biol. Med.* **44**, 1926–1934.
103. Pfitzner, M., J. C. Schlothauer, E. Bastien, S. Hackbarth, L. Bezdetnaya, H.-P. Lassalle and B. Röder (2016) Prospects of *in vivo* singlet oxygen luminescence monitoring: Kinetics at different locations on living mice. *Photodiag. Photodyn. Ther.* **14**, 204–210.
104. Snyder, J. W., E. Skovsen, J. D. C. Lambert, L. Poulsen and P. R. Ogilby (2006) Optical detection of singlet oxygen from single cells. *Phys. Chem. Chem. Phys.* **8**, 4280–4293.
105. Niedre, M. J., M. S. Patterson, A. Giles and B. C. Wilson (2005) Imaging of photodynamically generated singlet oxygen luminescence *in vivo*. *Photochem. Photobiol.* **81**, 941–943.
106. Kochevar, I. E. and R. W. Redmond (2000) Photosensitized production of singlet oxygen. *Methods Enzymol.* **319**, 20–28.
107. Hackbarth, S., S. Bornhütter and B. Röder (2016) Singlet oxygen in heterogeneous systems. In *Singlet Oxygen, Applications in Biosciences and Nanosciences*, Vol. **2** (Edited by S. Nonell and C. Flors), pp. 27–42. Royal Society of Chemistry, Cambridge, UK.
108. Walalawela, N. and A. Greer (2016) Remote singlet oxygen delivery strategies. In *Singlet Oxygen: Applications in Biosciences and Nanosciences*, Vol. **1** (Edited by S. Nonell and C. Flors), pp. 335–354. Royal Society of Chemistry, Cambridge, UK.
109. Kuimova, M. K., G. Yahioglu and P. R. Ogilby (2009) Singlet oxygen in a cell: Spatially dependent lifetimes and quenching rate constants. *J. Am. Chem. Soc.* **131**, 332–340.
110. Weil, J. A. and J. R. Bolton (2007) *Electron Paramagnetic Resonance: Elementary Theory and Practical Applications*, 2nd ed. John Wiley & Sons, Inc, Hoboken, New Jersey.
111. Janzen, E. G. (1971) Spin trapping. *Acc. Chem. Res.* **4**, 31–40.
112. Villamena, F. A. and J. L. Zweier (2004) Detection of reactive oxygen and nitrogen species by EPR spin trapping. *Antioxid. Redox Signal.* **6**, 619–629.
113. Thomas, A. H., M. P. Serrano, V. Rahal, P. Vicendo, C. Claparols, E. Oliveros and C. Lorente (2013) Tryptophan oxidation photosensitized by pterin. *Free Radic. Biol. Med.* **63**, 467–475.
114. Kang, P. and C. S. Foote (2002) Formation of transient intermediates in low-temperature photosensitized oxidation of an 8-^{13}C-guanosine derivative. *J. Am. Chem. Soc.* **124**, 4865–4873.
115. Steiner, U., G. Winter and H. E. A. Kramer (1977) Investigation of physical triplet quenching by electron donors. *J. Phys. Chem.* **81**, 1104–1110.
116. Terao, J., Y. Minami and N. Bando (2011) Singlet molecular oxygen-quenching activity of carotenoids: Relevance to protection of the skin from photoaging. *J. Clin. Biochem. Nutr.* **48**, 57–62.

117. Girotti, A. W. (2001) Photosenstitized oxidation of membrane lipids: Reaction pathways, cytotoxic effects, and cytoprotective mechanisms. *J. Photochem. Photobiol. B* **63**, 103–113.
118. Niziolek, M., W. Korytowski and A. W. Girotti (2005) Self-sensitized photodegradation of membrane-bound protoporphyrin mediated by chain lipid peroxidation: Inhibition by nitric oxide with sustained singlet oxygen damage. *Photochem. Photobiol.* **81**, 299–305.
119. Kang, P. and C. S. Foote (2000) Synthesis of a ^{13}C,^{15} N labeled imidazole and characterization of the 2,5-endoperoxide and its decomposition. *Tetrahedron Lett.* **41**, 9623–9626.
120. Ghimire, B., G. J. Lee, S. Mumtaz and E. H. Choi (2018) Scavenging effects of ascorbic acid and mannitol on hydroxyl radicals generated inside water by an atmospheric pressure plasma jet. *AIP Adv.* **8**, 075021.
121. Nosaka, Y. and A. Y. Nosaka (2017) Generation and detection of reactive oxygen species in photocatalysis. *Chem. Rev.* **117**, 11302–11336.
122. Nosaka, Y., Y. Yamashita and H. Fukuyama (1997) Application of chemiluminescent probe to monitoring superoxide radicals and hydrogen peroxide in TiO_2 photocatalysis. *J. Phys. Chem. B* **101**, 5822–5827.
123. Wrona, M., K. B. Patel and P. Wardman (2008) The roles of thiol-derived radicals in the use of 2′,7′-dichlorodihydrofluorescein as a probe for oxidative stress. *Free Radic. Biol. Med.* **44**, 56–62.
124. Chen, X., Z. Zhong, Z. Xu, L. Chen and Y. Wang (2010) 2′,7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy. *Free Radic. Res.* **44**, 587–604.
125. Karlsson, M., T. Kurz, U. T. Brunk, S. E. Nilsson and C. L. Frennesson (2010) What does the commonly used DCF test for oxidative stress really show? *Biochem. J.* **428**, 183–190.
126. Kalyanaraman, B., V. Darley-Usmar, K. J. Davies, P. A. Dennery, H. J. Forman, M. B. Grisham, G. E. Mann, K. Moore, L. J. Roberts 2nd and H. Ischiropoulos (2012) Measuring reactive oxygen and nitrogen species with fluorescent probes: Challenges and limitations. *Free Radic. Biol. Med.* **52**, 1–6.
127. Forman, H. J., O. Augusto, R. Brigelius-Flohe, P. A. Dennery, B. Kalyanaraman, H. Ischiropoulos, G. E. Mann, R. Radi, L. J. Roberts 2nd, J. Viña and K. J. Davies (2015) Even free radicals should follow some rules: A guide to free radical research terminology and methodology. *Free Radic. Biol. Med.* **78**, 233–235.
128. Zielonka, J., M. Zielonka, A. Sikora, J. Adamus, J. Joseph, M. Hardy, O. Ouari, B. P. Dranka and B. Kalyanaraman (2012) Global profiling of reactive oxygen and nitrogen species in biological systems: High-throughput real-time analyses. *J. Biol. Chem.* **287**, 2984–2995.
129. Sies, H., V. V. Belousov, N. S. Chandel, M. J. Davies, D. P. Jones, G. E. Mann, M. P. Murphy, M. Yamamoto and C. Winterbourn (2022) Defining roles of specific reactive oxygen species (ROS) in cell biology and physiology. *Nat. Rev. Mol. Cell Biol.* **23**, 499–515.
130. Gollmer, A., J. Arnbjerg, F. H. Blaikie, B. W. Pedersen, T. Beitenbach, K. Daasbjerg, M. Glasius and P. R. Ogilby (2011) Singlet oxygen sensor green®: Photochemical behavior in solution and in a mammalian cell. *Photochem. Photobiol.* **87**, 671–679.
131. Ruiz-Gonzalez, R., R. Bresoli-Obach, O. Gulias, M. Agut, H. Savoie, R. W. Boyle, S. Nonell and F. Giuntini (2017) NanoSOSG: A nanostructured fluorescent probe for the detection of intracellular singlet oxygen. *Angew. Chem. Int. Ed.* **56**, 2885–2888.
132. Yang, M., J. Zhang, D. Shabat, J. Fan and X. Peng (2020) Near-infrared chemiluminescent probe for real-time monitoring singlet oxygen in cells and mice model. *ACS Sens.* **5**, 3158–3164.
133. Kalyanaraman, B., B. P. Dranka, M. Hardy, R. Michalski and J. Zielonka (2014) HPLC-based monitoring of products formed from hydroethidine-based fluorogenic probes--the ultimate approach for intra- and extracellular superoxide detection. *Biochim. Biophys. Acta* **1840**, 739–744.
134. Zielonka, J., M. Zielonka and B. Kalyanaraman (2019) HPLC-based monitoring of oxidation of hydroethidine for the detection of NADPH oxidase-derived superoxide radical anion. *Methods Mol. Biol.* **1982**, 243–258.
135. Michalski, R., D. Thiebaut, B. Michałowski, M. M. Ayhan, M. Hardy, O. Ouari, M. Rostkowski, R. Smulik-Izydorczyk, A. Artelska, A. Marcinek, J. Zielonka, B. Kalyanaraman and A. Sikora (2020) Oxidation of ethidium-based probes by biological radicals: Mechanism, kinetics and implications for the detection of superoxide. *Sci. Rep.* **10**, 18626.
136. Sikora, A., J. Zielonka, K. Dębowska, R. Michalski, R. Smulik-Izydorczyk, J. Pięta, R. Podsiadły, A. Artelska, K. Pierzchała and B. Kalyanaraman (2020) Boronate-based probes for biological oxidants: A novel class of molecular tools for redox biology. *Front. Chem.* **8**, 580899.
137. Di Mascio, P., G. R. Martinez, S. Miyamoto, G. E. Ronsein, M. H. G. Medeiros and J. Cadet (2016) Singlet molecular oxygen: Düsseldorf – São Paulo, the Brazilian connection. *Arch. Biochem. Biophys.* **595**, 161–175.
138. Miyamoto, S., G. R. Martinez, A. P. B. Martins, M. H. G. Medeiros and P. Di Mascio (2003) Direct evidence of singlet molecular oxygen [O_2 ($^1\Delta_g$)] production in the reaction of linoleic acid hydroperoxide with peroxynitrite. *J. Am. Chem. Soc.* **125**, 4510–4517.
139. Miyamoto, S., G. R. Martinez, M. H. G. Medeiros and P. Di Mascio (2003) Singlet molecular oxygen generated from lipid hydroperoxides by the Russell mechanism: Studies using ^{18}O-labeled linoleic acid hydroperoxide and monomol light emission measurements. *J. Am. Chem. Soc.* **125**, 6172–6179.
140. Ravanat, J.-L., G. R. Martinez, M. H. G. Medeiros, P. Di Mascio and J. Cadet (2006) Singlet oxygen oxidation of 2′-deoxyguanosine. Formation and mechanistic insights. *Tetrahedron* **62**, 10709–10715.
141. Martinez, G. R., J.-L. Ravanat, M. H. G. Medeiros, J. Cadet and P. Di Mascio (2000) Synthesis of a naphthalene endoperoxide as a source of ^{18}O-labeled singlet oxygen for mechanistic studies. *J. Am. Chem. Soc.* **122**, 10212–10213.
142. Braun, A. M., H. Dahn, E. Gassmann, I. Gerothanassis, L. Jakob, J. Kateva, C. G. Martinez and E. Oliveros (1999) (2 + 4)-cycloaddition with singlet oxygen. ^{17}O-Investigation of the reactivity of furfuryl alcohol endoperoxide. *Photochem. Photobiol.* **70**, 868–874.
143. Kang, P. and C. S. Foote (2002) Photosensitized oxidation of ^{13}C,^{15}N-labeled imidazole derivatives. *J. Am. Chem. Soc.* **124**, 9629–9638.
144. Cadet, J., T. Douki and J.-L. Ravanat (2008) Oxidatively generated damage to the guanine moiety of DNA: Mechanistic aspects and formation in cells. *Acc. Chem. Res.* **41**, 1075–1083.
145. Cadet, J., K. J. A. Davies, M. H. Medeiros, P. Di Mascio and J. R. Wagner (2017) Formation and repair of oxidatively generated damage in cellular DNA. *Free Radic. Biol. Med.* **107**, 13–34.
146. Misiaszek, R., C. Crean, A. Joffe, N. E. Geacintov and V. Shafirovich (2004) Oxidative DNA damage associated with combination of guanine and superoxide radicals and repair mechanisms via radical trapping. *J. Biol. Chem.* **279**, 32106–32115.
147. Cadet, J., M. Berger, G. W. Buchko, P. C. Joshi, S. Raoul and J.-L. Ravanat (1994) 2,2-Diamino-4-[(3,5-di-O-acetyl-2-seoxy-β-D-erythro-pentofuranosyl)amino]-5-(2*H*)-oxazolone: A novel and predominant radical oxidation product of 3′,5′-di-*O*-acetyl-2′-deoxyguanosine. *J. Am. Chem. Soc.* **116**, 7403–7404.
148. Kino, K., I. Saito and H. Sugyiama (1998) Product analysis of GG specific photooxidation of DNA via electron transfer: 2-aminoimidazolone as a major guanine oxidation product. *J. Am. Chem. Soc.* **120**, 7373–7474.
149. Gasparutto, D., J.-L. Ravanat, O. Gerot and J. Cadet (1998) Synthesis, characterization and chemical stability of oligonucleotides containing 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl) amino]-5(2*H*)-oxazolone. *J. Am. Chem. Soc.* **120**, 10283–10286.
150. Haraguchi, K., M. O. Delaney, C. J. Wiederholt, A. Sambandam, Z. Hantosi and M. M. Greenberg (2002) Synthesis and characterization of oligodeoxynucleotides containing formamidopyrimidine lesions and nonhydrolyzable analogues. *J. Am. Chem. Soc.* **124**, 3263–3269.
151. Cadet, J., S. Loft, R. Olinski, M. D. Evans, K. Bialkowski, J. R. Wagner, P. C. Dedon, P. Møller, M. M. Greenberg and M. S. Cooke (2012) Biologically relevant oxidants and terminology, classification and nomenclature of oxidatively generated damage to nucleobases and 2-deoxyribose in nucleic acids. *Free Radic. Res.* **46**, 367–381.

152. Ravanat, J.-L., C. Saint-Pierre, P. Di Mascio, G. R. Martinez, M. H. G. Medeiros and J. Cadet (2001) Damage to isolated DNA mediated by singlet oxygen. *Helv. Chim. Acta* **84**, 3702–3709.
153. Cadet, J. and T. Douki (2019) Formation of UV-induced DNA damage contributing to skin cancer development. *Photochem. Photobiol. Sci.* **17**, 1816–1841.
154. Luo, W. C., J. G. Muller, E. M. Rachlin and C. J. Burrows (2000) Characterization of spiroiminodihydantoin as a product of one-electron oxidation of 8-oxo-7,8-dihydroguanosine. *Org. Lett.* **2**, 613–616.
155. Ravanat, J.-L., C. Saint-Pierre and J. Cadet (2003) One-electron oxidation of the guanine moiety of 2′-deoxyguanosine: Influence of 8-oxo-7,8-dihydro-2′-deoxyguanosine. *J. Am. Chem. Soc.* **125**, 2030–2031.
156. Niles, J. C., J. S. Wishnok and S. R. Tannenbaum (2001) Spiroiminodihydantoin is the major product of the 8-oxo-7,8-dihydroguanosine reaction with peroxynitrite in the presence of thiols and guanosine photooxidation by methylene blue. *Org. Lett.* **3**, 963–966.
157. Prat, F., K. N. Houk and C. S. Foote (1998) Effects of guanine stacking on the oxidation of 8-oxoguanine in β-DNA. *J. Am. Chem. Soc.* **120**, 845–846.
158. Bernstein, R., F. Prat and C. S. Foote (1999) On the mechanism of DNA cleavage by fullerenes investigated in model systems: Electron transfer from guanosine and 8-oxo-guanosine derivatives to C_{60}. *J. Am. Chem. Soc.* **121**(46), 4–465.
159. Luo, W., J. G. Muller and C. J. Burrows (2001) The pH-dependent role of superoxide in riboflavin-catalyzed photooxidation of 8-oxo-7,8-dihydroguanosine. *Org. Lett.* **3**, 2801–2804.
160. Cadet, J., S. Bellon, M. Berger, A. G. Bourdat, T. Douki, V. Duarte, S. Frelon, D. Gasparutto, E. Muller, J.-L. Ravanat and S. Sauvaigo (2002) Recent aspects of oxidative DNA damage: Guanine lesions, measurement and substrate specificity of DNA repair glycosylases. *Biol. Chem.* **383**, 933–943.
161. Frelon, S., T. Douki, J.-L. Ravanat, J.-P. Pouget, C. Tornabene and J. Cadet (2000) High-performance liquid chromatography--tandem mass spectrometry measurement of radiation-induced base damage to isolated and cellular DNA. *Chem. Res. Toxicol.* **13**, 1002–1010.
162. Berger, M. and J. Cadet (1985) Isolation and characterization of the radiation-induced degradation products of 2′-deoxyguanosine in oxygen-free aqueous solutions. *Z. Naturforsch. B* **40**, 1519–1531.
163. Douki, T., R. Martini, J.-L. Ravanat, R. J. Turesky and J. Cadet (1997) Measurement of 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 8-oxo-7,8-dihydroguanine in isolated DNA exposed to gamma radiation in aqueous solution. *Carcinogenesis* **18**, 2385–2391.
164. Douki, T. and J. Cadet (1999) Modification of DNA bases by photosensitized one-electron oxidation. *Int. J. Radiat. Biol.* **75**, 571–581.
165. Perrier, S., J. Hau, D. Gasparutto, J. Cadet, A. Favier and J.-L. Ravanat (2006) Characterization of lysine-guanine cross-links upon one-electron oxidation of a guanine-containing oligonucleotide in the presence of a trilysine peptide. *J. Am. Chem. Soc.* **128**, 5703–5710.
166. Maxam, A. M. and W. Gilbert (1980) Sequencing and labeled DNA with base-specific chemical cleavage. *Methods Enymol.* **65**, 499–560.
167. Ito, K., S. Inoue, K. Yamamoto and S. Kawanishi (1993) 8-Hydroxydeoxyguanosine formation at the 5′ site of 5′-GG-3′ sequences in double-stranded DNA by UV radiation with riboflavin. *J. Biol. Chem.* **268**, 13221–13227.
168. Hiraku, Y. and S. Kawanishi (2000) Distinct mechanisms of guanine specific DNA photodamage induced by nalidixic acid and fluoroquinolone antibacterials. *Arch. Biochem. Biophys.* **382**, 211–218.
169. Hiraku, Y., K. Ito, K. Hirakawa and S. Kawanishi (2007) Photosensitized DNA damage and its protection via a novel mechanism. *Photochem. Photobiol.* **83**, 205–212.
170. Hirakawa, K., K. Ota, J. Hirayama, S. Oikawa and S. Kawanishi (2014) Nile blue can photosensitize DNA damage through electron transfer. *Chem. Res. Toxicol.* **27**, 649–655.
171. Meniel, V. and R. Waters (1999) Spontaneous and photosensitiser-induced DNA single-strand breaks and formamidopyrimidine-DNA glycosylase sensitive sites at nucleotide resolution in the nuclear and mitochondrial DNA of *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **27**, 822–830.
172. Ito, K. and S. Kawanishi (1997) Photoinduced hydroxylation of deoxyguanosine in DNA by pterins. Sequence specificity and mechanism. *Biochemistry* **36**, 1774–17781.
173. Sugiyama, H. and I. Saito (1996) Theoretical studies of GG specific cleavage of DNA via electron transfer: Significant lowering of ionization potential and 5′-localization of HOMO of stacked GG bases in B-form DNA. *J. Am. Chem. Soc.* **118**, 7063–7068.
174. Yoshioka, Y., Y. Kitagawa, Y. Takano, K. Yamaguchi, T. Nakamura and I. Saito (1999) Experimental and theoretical studies on the selectivity of GGG triplets toward one-electron oxidation in B-form of DNA. *J. Am. Chem. Soc.* **121**, 8712–8719.
175. Kasai, H., Z. Yamaizumi, M. Berger and J. Cadet (1992) Photosensitized formation of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-fydroxy-2′-deoxyguanosine) in DNA by riboflavin: A nonsinglet oxygen mediated reaction. *J. Am. Chem. Soc.* **114**, 9692–9694.
176. Martinez, G. R., J.-L. Ravanat, J. Cadet, M. H. de Medeiros and P. Di Mascio (2007) Spiroiminodihydantoin nucleoside formation from 2′-deoxyguanosine oxidation by [^{18}O-labeled] singlet molecular oxygen in aqueous solution. *J. Mass Spectrom.* **42**, 1326–1332.
177. Cadet, J., J. R. Wagner and D. Angelov (2019) Biphotonic ionization of DNA: From model studies to cell. *Photochem. Photobiol.* **95**, 59–72.
178. Valencia, A. and I. E. Kochevar (2008) Nox1-based NADPH oxidase is the major source of UVA-induced reactive oxygen species in human keratinocytes. *J. Invest. Dermatol.* **128**, 214–222.
179. Cadet, J., T. Douki and J.-L. Ravanat (2015) Oxidatively generated damage to cellular DNA by UVB and UVA radiation. *Photochem. Photobiol.* **91**, 140–155.
180. Cadet, J., D. Angelov and J. R. Wagner (2022) Hydroxyl radical is predominantly involved in oxidatively generated base damage to cellular DNA exposed to ionizing radiation. *Int. J. Radiat. Biol.*, 1–7.
181. Collins, A. R. (2014) Measuring oxidative damage to DNA and its repair with the comet assay. *Biochim. Biophys. Acta* **1840**, 794–800.
182. Møller, P., K. Jantzen, M. Løhr, M. H. Andersen, D. M. Jensen, M. Roursgaard, P. H. Danielsen, A. Jensen and S. Loft (2018) Searching for assay controls for the Fpg- and hOGG1-modified comet assay. *Mutagenesis* **33**, 9–19.
183. Tyrrell, R. M. and M. Pidoux (1989) Singlet oxygen involvement in the inactivation of cultured human fibroblasts by UVA (334 nm, 365 nm) and near-visible (405 nm) radiations. *Photochem. Photobiol.* **49**, 407–412.
184. Tyrrell, R. M. and S. M. Keyse (1990) New trends in photobiology. The interaction of UVA radiation with cultured cells. *J. Photochem. Photobiol. B* **4**, 349–361.
185. Kielbassa, C., L. Roza and B. Epe (1997) Wavelength dependence of oxidative DNA damage induced by UV and visible light. *Carcinogenesis* **18**, 811–816.
186. Kielbassa, C. and B. Epe (2000) DNA damage induced by ultraviolet and visible light and its wavelength dependence. *Methods Enzymol.* **319**, 436–445.
187. Cadet, J., E. Sage and T. Douki (2005) Ultraviolet radiation mediated damage to cellular DNA. *Mutat. Res.* **571**, 3–17.
188. Courdavault, S., C. Baudouin, M. Charveron, A. Favier, J. Cadet and T. Douki (2004) Larger yield of cyclobutane dimers than 8-oxo-7,8-dihydroguanine in the DNA of UVA-irradiated human skin cells. *Mutat. Res.* **556**, 135–142.
189. Mouret, S., C. Baudouin, M. Charveron, A. Favier, J. Cadet and T. Douki (2006) Cyclobutane pyrimidine dimers are predominant DNA lesions in whole human skin exposed to UVA radiation. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 13765–13770.
190. Mouret, S., A. Forestier and T. Douki (2012) The specificity of UVA induced DNA damage in human melanocytes. *Photochem. Photobiol. Sci.* **11**, 155–162.
191. Kvam, E. and R. M. Tyrrell (1999) The role of melanin in the induction of oxidative DNA Base damage by ultraviolet a irradiation of DNA or melanoma cells. *J. Invest. Dermatol.* **113**, 209–213.
192. Szewczyk, G., A. Zadlo, M. Sarna, S. Ito, K. Wakamatsu and T. Sarna (2016) Aerobic Photoreactivity of synthetic Eumelanins and Pheomelanins: Generation of singlet oxygen and superoxide anion. *Pigment Cell Melanoma Res.* **29**, 669–678.

193. Chiarelli-Neto, O., A. S. Ferreira, W. K. Martins, C. Pavani, D. Severino, F. Faiao-Flores, S. S. Maria-Engler, E. Aliprandini, G. R. Martinez, P. Di Mascio, M. H. Medeiros and M. S. Baptista (2014) Melanin photosensitization and the effect of visible light on epithelial cells. *PLoS One* **9**, e113266.
194. McBride, T. J., J. E. Schneider, R. A. Floyd and L. A. Loeb (1992) Mutations induced by methylene blue plus light in single-stranded M13mp2. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6866–6870.
195. Wagner, J. R. and J. Cadet (2010) Oxidation reactions of cytosine DNA components by hydroxyl radical and one-electron oxidants in aerated aqueous solutions. *Acc. Chem. Res.* **43**, 564–571.
196. Cadet, J., C. Decarroz, S. Y. Wang and W. R. Midden (1983) Mechanisms and products of photosensitized degradation of nucleic acids and related model compounds. *Isr. J. Chem.* **23**, 420–429.
197. Tuite, E. M. and J. M. Kelly (1993) Photochemical interactions of methylene blue and analogues with DNA and other biological substrates. *J. Photochem. Photobiol. B* **21**, 103–124.
198. Berra, C. M., C. S. de Oliveira, C. C. Garcia, C. R. Rocha, L. K. Lerner, L. C. Lima, M. S. Baptista and C. F. M. Menck (2013) Nucleotide excision repair activity on DNA damage induced by photoactivated methylene blue. *Free Radic. Biol. Med.* **61**, 343–356.
199. Pouget, J.-P., T. Douki, M.-J. Richard and J. Cadet (2000) DNA damage induced in cells by gamma and UVA radiation as measured by HPLC/GC-MS and HPLC-EC and comet assay. *Chem. Res. Toxicol.* **13**, 541–549.

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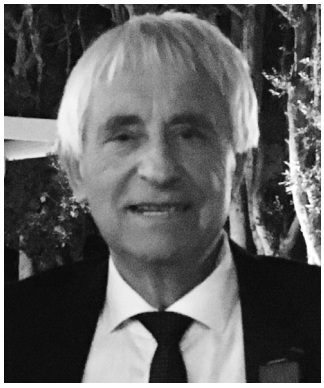

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