Chapter 2
Photophysical and Photochemical Mechanisms

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Abstract Photodynamic therapy (PDT) harnesses the power of light in an elegant method to produce cytotoxic agents in a spatially and temporally controlled manner and specifically damage target cells and tissues. For photodynamic reactions to occur, the PS molecule must absorb at least one photon to be promoted to a sufficiently long-lived excited state and then induce photodynamic reactions in an oxygenated environment. Such properties guarantee that PDT has an exceptionally broad action spectrum against tumors or pathogens, and resistance occurrence is restricted to only a few exceptions that can be avoided using simple strategies. To fully understand the intricacies of the mechanisms by which PDT acts, it is clear that one must take advantage of all the basic sciences (e.g., physics, chemistry, and biology). In fact, such conceptual complexity still maintains constant scientific investigations to deeply understand the molecular basis of PDT. Curiously, it might also be one of the reasons to explain why this hundred-year-old technique is still not generally applied in clinics or taught in standard courses of pharmacology. In this

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chapter, we will attempt to use a multidisciplinary approach, with simple technical language and a minimum of mathematics and equations, to allow any student with minimal training in basic sciences to understand all the fundamental mechanisms of PDT.

2.1 Introduction

Ever since the very first moments following the Big Bang cosmological event, electromagnetic radiation (EMR) has spread through the whole universe introducing various degrees of perturbations into the properties of condensed matter. Our planet Earth was formed and evolved continuously receiving large amounts of radiation from our closest star, the Sun. Human kind has recognized long ago that life is impossible without sunlight and in diverse historical periods has worshiped the Sun, or a personification of it, as a powerful god. During daylight primitive people used light to search for food and escape from predators, and during the long and warm days of summer, living became easier and food was much more abundant. In fact, current scientific knowledge proposes that all life on the Earth evolved under the influence sunlight, which induced the formation of the very first organic molecules in the atmosphere or oceans [1].

At some point, living organisms developed the capacity to use photosynthesis, which became a complex series of physicochemical reaction processes to harness energy from sunlight to produce the basic organic compounds needed for cellular metabolism. Over billions of years, all life thrived in such a "radiation-rich" environment forming a plethora of variations in the food chain all of which depended on photosynthesis either directly or indirectly. However, photosynthesis is far from being the only physicochemical processes based on absorption of energy from light that life-forms have evolved to relay on. Light also represents the means we use to interact with the environment (e.g., different forms of vision), regulates our circadian rhythms through night and day cycles, and catalyzes the production of micronutrients (e.g., vitamin D). Light can be a source of cellular damage (e.g., erythema induced by ultraviolet and DNA damage caused by X-rays, gamma rays, and UV and even thermal effects caused by infrared radiation), but light can also cause the repair of damage to biomolecules and tissues (e.g., photolyase enzyme, photobiomodulation therapy).

Pigmentation seems to play an important role in the animal kingdom as a protective defense against radiation. It is well known that solar erythema affects animals, such as cattle and horses, almost exclusively in the lightly pigmented areas, while the darker parts are less affected. Hence, the anatomical regions most exposed to the sun are usually more deeply pigmented than less sun-exposed regions. Examples of this are found almost everywhere: among furred animals, whales, reptiles, birds, fish, etc. These phenomena are not very different from what we see in humans. Due to geographic dispersal during human evolution, populations inhabiting regions

close to the equator present more strongly pigmented skin than those who evolved in regions closer to the polar regions. Yet, people with darker skin tones still show much lighter pigmentation in regions less exposed to the sun, such as the palms of hands and soles of the feet.

Nowadays it is well known that some wavelengths, or "colors", of visible and near-ultraviolet (NUV) light can damage and even kill bacteria. In the late 1800s, researchers performed several investigations and concluded that sunlight was the best, cheapest, and most universally applicable antimicrobial agent we have [2]. Back then, the NUV and blue regions (300–500 nm) of the EMR spectrum were referred as chemical rays because the chemical effects were more pronounced and the heating effects were at a minimum. Using dyes, crystals, and prisms to separate light from the sun into monochromatic colors, the so-called chemical rays were determined to be the most effective in killing bacteria and causing erythema than the opposite end of the spectrum, the infrared "thermal rays." It was then realized that cellular damage and inactivation induced by sunlight was more closely related to photochemical reactions than photothermal effects. However, the photochemical mechanisms behind such effects would only be understood about half a century later, even though Niels Finsen was awarded a Nobel Prize in the meantime (see Chap. 1). Currently, it is known that EMR from sunlight can directly damage biomolecules through ionization (e.g., production of free radicals, photodimerization of thymine residues in DNA) but also acts indirectly through excitation of endogenous photosensitizers (PS) (e.g., riboflavin and metal-free porphyrins) present in cells producing reactive oxygen species (ROS) through photodynamic reactions [3].

Photodynamic therapy (PDT) harnesses the power of light in an elegant method to produce cytotoxic agents in a spatially and temporally controlled manner and specifically damage target cells and tissues. For photodynamic reactions to occur, the PS molecule must absorb at least one photon to excite its electronic state as predicted by quantum theory. PS compounds with a sufficiently long-lived excited state can then induce photodynamic reactions that are directly or indirectly dependent on the presence of O_2 to locally form free radicals and singlet oxygen ($^1O_2$). Since many PS are nontoxic in the dark and can preferentially accumulate in tumors or pathogens, only minor side effects are expected. However, when the PS-loaded cells are illuminated, multiple relevant biomolecules can be rapidly degraded. Such properties guarantee that PDT has an exceptionally broad action spectrum against tumors or pathogens, and resistance occurrence is restricted to only a few exceptions that can be avoided using simple strategies.

To fully understand the intricacies of the mechanisms by which PDT acts, it is clear that one must take advantage of all the basic sciences (e.g., physics, chemistry, and biology). In fact, such conceptual complexity is still a subject of study and might be one of the reasons to explain why this hundred-year-old technique is still not generally applied in clinics or taught in standard courses of pharmacology. In this chapter, we will attempt to use a multidisciplinary approach, with simple technical language and a minimum of mathematics and equations, to allow any student with minimal training in basic sciences to understand all the fundamental mechanisms of PDT.

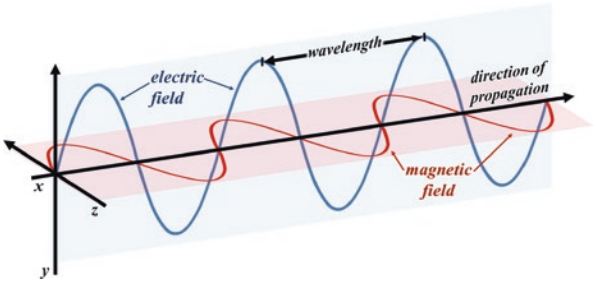

Fig. 2.1 Schematic illustration of a classic electromagnetic wave. It consists of transverse oscillations of electric and magnetic fields that are perpendicular to each other and in relation to the propagation (motion) direction. Wavelength is determined by the distance between two peaks or valleys. The speed of light in vacuum is $c_0 = 299{,}792{,}458$ m/s

2.2 Electromagnetic Radiation

Before presenting the biological effects of PDT, this chapter will introduce the nature of light and how it interacts with the electrons present in atoms and molecules to induce physical and chemical effects that will eventually lead to photodynamic reactions. The term electromagnetic radiation refers to a certain kind of energy that propagates through space (what used to be called the "ether"), and which can be interpreted similarly to heat being considered the propagation of vibrational energy through solid material, or wind as being the propagation of kinetic energy through air. However, this form of radiant energy, or radiation, can propagate in absolute vacuum at the highest speed theoretically possible in nature: the speed of light, $\mathbf{c_0} = 299{,}792{,}458$ meters per second (m/s). The question of whether EMR is better interpreted as a wave or as a particle (i.e., photon) has spurred exhaustive discussions among scientists, over the past few centuries. Only with the advent of quantum mechanics and precise experiments carried out over the last century has it been possible to demonstrate that both interpretations were equally valid for diverse situations. Depending on the physical observation, EMR can either be interpreted as a wave or as a particle carrying a discrete amount, or "quantum," of energy. In fact, Louis de Broglie postulated in 1924 the "wave-particle duality theory" proposing that any accelerated particle is associated with a wave, and vice versa (any wave is associated with a particle), thus redefining the whole concept of radiation itself [4]. Under his theoretical interpretation, the kinetic energy of any moving particle with mass, such as protons and electrons in particle accelerators, can be directly related to a certain wavelength of corpuscular radiation. For the purpose of this book, however, we will retain our attention only to massless and charge-free particles of EMR called "photons."

EMR is classically defined as transverse waves with oscillations of electric and magnetic fields that are perpendicular to each other and both perpendicular to the direction of propagation (Fig. 2.1). As any wave propagating at a certain velocity ($\boldsymbol{V}$), EMR can be described in terms of specific wavelength ($\boldsymbol{\lambda}$) and frequency (ν) of

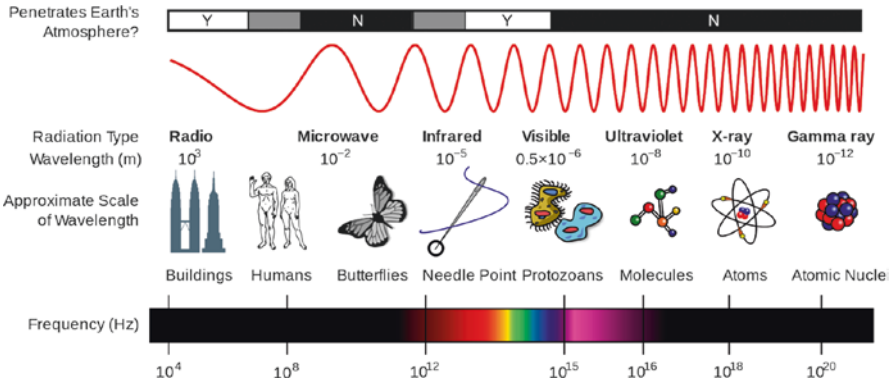

Fig. 2.2 Diagram of electromagnetic spectrum illustrating various properties across a broad range of wavelengths or frequencies. Note that only some regions of radio waves and visible light can transpose the current Earth's atmosphere and reach the ground (Image was modified from Inductive load, at Wikipedia. This image has "GNU Free Documentation License")

oscillation (Eq. 2.1). According to the international system of units, λ is measured in meters (m) and ν in Hertz (Hz, i.e., number of oscillations per second). In some circumstances, the wave number ($\bar{\omega}$, i.e., number of waves per centimeter) is alternatively used to characterize EMR. Modern physics describes EMR as a photon particle where the exact quantum of energy (E) of each individual photon can be easily calculated in respect to its frequency times the Planck constant ($h = 6.6260700 \times 10^{-34}$ J.s), as presented in Eq. (2.2). Hence, according to Eqs. (2.1 and 2.2), in lower wavelengths or higher frequencies, the photon energy becomes higher:

$$V = c_0 = \lambda \upsilon \tag{2.1}$$

$$E = h\upsilon = \frac{h.c_0}{\lambda} \tag{2.2}$$

The energy associated with EMR has a variation of many orders of magnitude across the whole spectrum (Fig. 2.2). According to increasing levels of frequency or energy, the electromagnetic radiation is classified into radio waves, microwaves, infrared radiation, visible light, ultraviolet radiation, X-rays, and gamma rays. According to Fig. 2.2, for example, one single gamma ray photon can carry up to 100,000 times the energy of a single visible light photon. Therefore, a gamma ray photon can induce physical phenomena in atoms or molecules that require 100,000 times the energy of a visible light photon. Conversely, a gamma ray photon would have 10^{15} times as much energy as a radio wave photon.

The physical phenomena that occur in matter associated with the emission or absorption of a photon are directly proportional to its energy. The less energetic radio- and microwave photons induce rotation of molecules and atoms to increase their kinetic energy as they spin around their center of mass. Electricity-conducting

materials, such as metals, also allow electron translation and produce electric currents. Infrared radiation, at wavelengths from 1 to 1000 μm, increases the kinetic energy of molecules by means of vibration. The chemical bounds within molecules are not rigid. They behave mostly as flexible springs constantly oscillating at vibrational modes of certain frequencies and coordination (e.g., stretching, bending, wagging, twisting, etc.). Each different type of molecular bond has characteristic vibrational modes that can be promoted by infrared photons of matching energy. Since temperature is a measure of the average kinetic energy of particles of matter, such as atoms and molecules, the absorption of these types of radiation by biological systems is mostly related to heat induction alone and often leads to nonspecific thermal effects on biological systems. On the opposite extremity of the EMR spectrum, there are X-rays and gamma rays with very high-energy photons that remove electrons from inner electronic shells of atoms or molecules and produce free radicals and ions. We commonly refer them as ionizing radiation due to this strong characteristic. This radiation can severely damage living organisms by direct and indiscriminate ionization of biomolecules, allied to simultaneous formation of free radicals originating from broken bonds. Ionized molecules become extremely reactive, and chemical bonds may be consequently broken or formed. Since absorption of these high-energy photons is generally nonspecific, the ability to "target" molecules is poor and constantly leads to the damaging side effects of radiotherapy.

Only a small portion of the EMR spectrum can be used to target the excitation (but not removal) of electrons in specific molecules with satisfactory precision. This region encompasses near-UV, visible, and near-infrared radiation (i.e., λ approximately from 300 to 900 nm). Since photodynamic reactions are governed by absorption of photons within this range, in this book the term "light" will always be used to mean this range of the EMR spectrum. Differently from other types of radiation we mentioned before, light interacts with electrons from valence shells of molecules or atoms that often absorb very characteristic wavelengths. As an example of such specificity, all vivid colors we can see are within the tiny interval of whole EMR spectrum known as visible radiation. A combination of red (600–700 nm), green/yellow (500–600 nm), and blue (400–500 nm) light is detected by retinal cones in our eyes and interpreted by our brain. Color-blind individuals are unable to see one or more of these colors because some of their retinal cones are absent or defective. Since what we detect is visible light that was either reflected, refracted, or emitted by some material, all color differences are relative to light absorption and scattering properties of each material. Take plant chlorophyll as an example: when white sunlight interacts with leafs, red and blue light are strongly absorbed, while all the green light is reflected and can be detected by our eyes. Note in Fig. 2.3 how small molecular alterations, such as a carboxyl group addition (red arrow), can shift light absorption bands among chlorophylls *a* and *b*. Chlorin e6 is a chlorophyll derivative molecular framework used as PS for PDT. The exclusion of the lipid radical and chelated Mg^{++} in chlorin e6 introduces further absorption shifts in relation to chlorophylls.

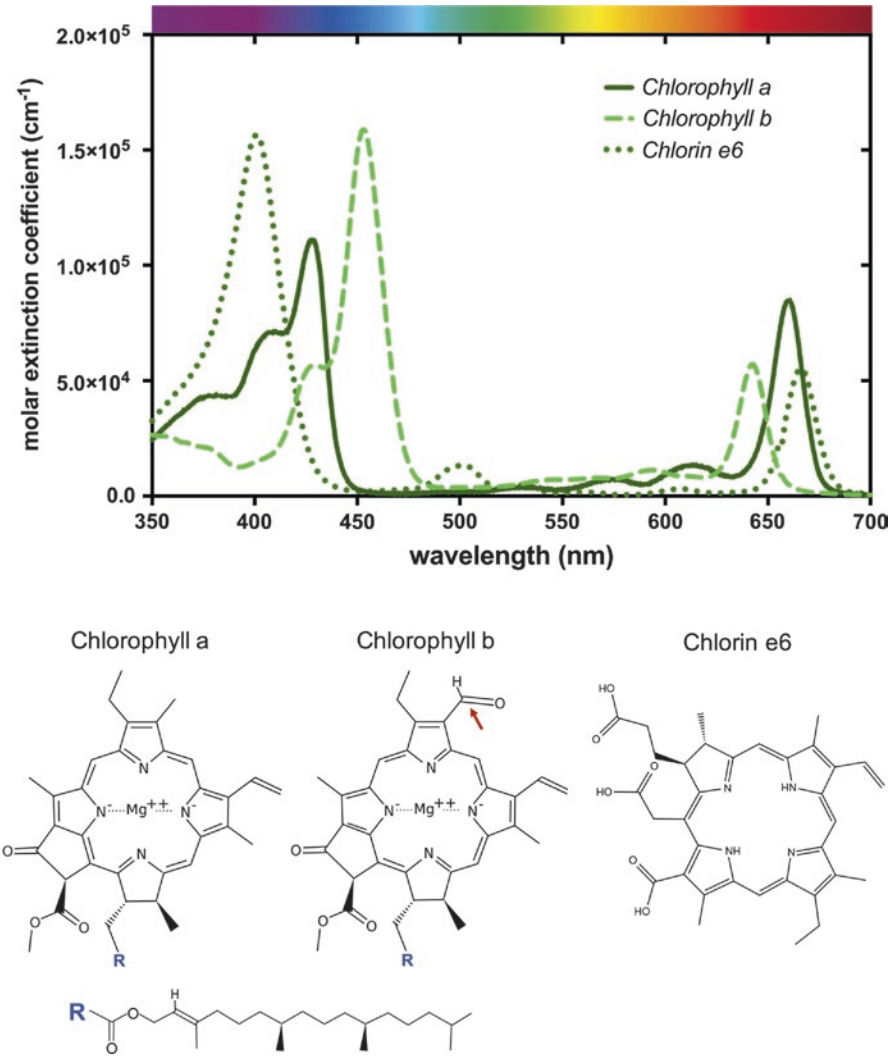

Fig. 2.3 Absorption spectra of three *green* pigments derived from plants: chlorophyll a, chlorophyll b, and chlorin (e6) (a chlorophyll derivative used as a photosensitizer for PDT). Absorption intensity is presented as molar extinction coefficient units in vertical axis, and wavelength is plotted in horizontal axis. *Colored bar* at the *top* illustrates the regions of visible light spectrum. Below are presented their respective molecular structures. Observe how small molecular alterations, such as a carbonyl group addition (*red arrow*), can shift light absorption bands among chlorophyll a and b (Data acquired from omlc.org/spectra/PhotochemCAD/index.html [13])

2.3 Photophysics and Photochemistry

The first law of photochemistry states that light must be absorbed by a molecule in order to induce a photochemical reaction. The second law of photochemistry completes the concept of light absorption, stating that each photon absorbed by a chemical system can only excite a single molecule. Therefore, light absorption is a fundamental process that must be understood by those who intend to explore the world of PDT. Each photosensitive molecular structure possesses a characteristic probability of photon absorption at different regions of the EMR spectrum. This is commonly called an "absorption spectrum." The Lambert-Beer law proposes a simple exponential equation (2.3) to measure the monochromatic light absorption efficiency of photosensitive molecules dissolved in a transparent solvent:

$$I = I_0 \times 10^{-\varepsilon lc} \tag{2.3}$$

where $\boldsymbol{I_0}$ is the initial light beam intensity and $\boldsymbol{I}$ is the light intensity transmitted through a medium of path length $\boldsymbol{l}$ with an absorbing compound dissolved at concentration $\boldsymbol{c}$. The term $\boldsymbol{\varepsilon}$ is a characteristic constant of each compound, named the "molar extinction coefficient," that tells us how efficient a molecule is to absorb light at that wavelength (usually the wavelength of peak absorption λ_{max}). Efficient PS for PDT commonly have absorption peaks with $\boldsymbol{\varepsilon}$ values greater than 10,000 $M^{-1}cm^{-1}$. This quantity means that light intensity at a certain wavelength can be decreased by 10,000 times after propagating through 1 centimeter of this photosensitizer solution at 1 molar of concentration. Even though light absorption during PDT applications can be enhanced by increasing the PS concentration, this approach must be taken carefully since high concentrations of PS can be toxic in the dark and lead to undesirable side effects or diminished therapy specificity. Moreover, very high local concentrations of PS can reduce light transmission into the tissue.

When a "ground-state molecule" (i.e., not an excited molecule) absorbs one photon of light, one valence shell electron can be promoted from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) to form an "excited-state molecule." To absorb a photon, the energy gap between the ground- and excited-state molecule (HOMO-LUMO gap) must match the quantum of energy carried by the photon. The extra amount of energy brought by a photon significantly alters the electronic configuration of valence shells transforming the excited-state molecules into new chemical species that have their own physical and chemical properties, which can be very different from those of ground-state molecules. Excited-state molecules, however, are usually unstable with a very short lifetime and may quickly return to their ground state via various possible deactivating processes (Fig. 2.4). Those can be photophysical processes (with or without photon emission), or photochemical reactions leading to production of new molecular species, such as ROS generated by photodynamic reactions. Each photosensitive molecule has characteristic probability to undergo each process, and it can be influenced by solvents and other molecules present in its surroundings.

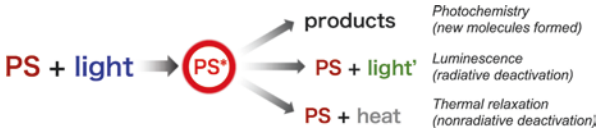

Fig. 2.4 Self-deactivation processes of photosensitizer (PS) molecules excited by light. Photochemical processes form new molecular species through isomerization, cleavage, synthesis, and electron or energy donation. The last two are predominant in photodynamic reactions. Photophysical processes can lead to luminescence through fluorescence or phosphorescence or heat generation through thermal relaxation (i.e., energy is transformed into kinetic energy)

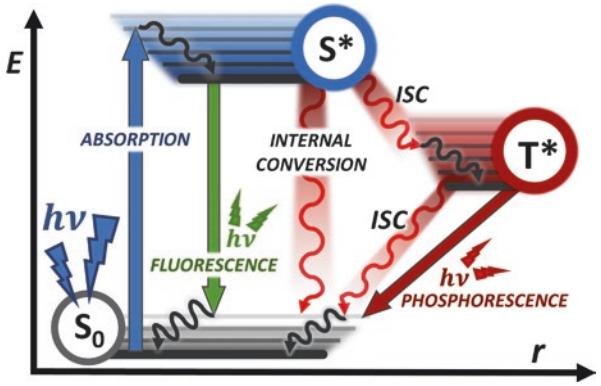

Fig. 2.5 Jablonski diagram illustration of possible photophysical processes. *Straight lines* represent radiative transitions and wavy lines nonradiative transitions. After photon absorption, ground-state singlet molecule is promoted to excited-state singlet and may decay back to ground state through fluorescence or internal conversion. Alternatively, S* may decay to a more stable triplet state through intersystem crossing (ISC) and only then be deactivated through phosphorescence or another ISC

There is a thin line that separates the concepts of photophysical and photochemical phenomena. To put it simply, photophysicists mostly deal with molecular and atomic excitation processes, including photon absorption itself, that lead to photon emission or thermal effects, but in general they do not focus on chemical reactions produced by excited states or the formation of new ground-state species. Of course, nowadays, many scientists work in multidisciplinary surroundings and researchers must consider all perspectives.

The "Jablonski diagram" (Fig. 2.5) is often used to conveniently illustrate such phenomena. It is can be interpreted as a map of physical and chemical events related to the interaction of light that can occur with a molecule. The electronic states are vertically separated in groups of parallel lines to illustrate the relative energies. Each line represents a particular vibrational mode of each state. In between the lines, there are multiple energy levels of rotational states. Radiative transitions, either by absorption or emission of a photon (***hν***), are represented in straight lines and nonradiative transitions as wavy lines. Excited-state molecules are indicated by asterisks (*). When a molecule is excited by light, an electron is promoted to higher

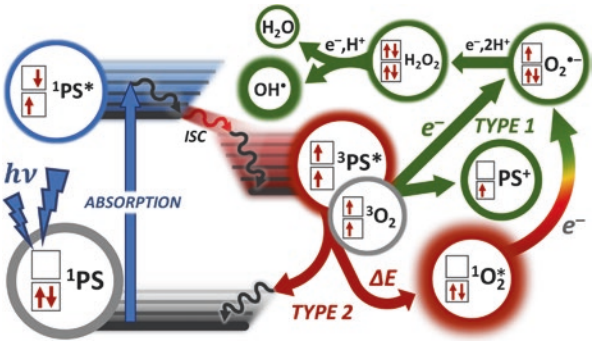

Fig. 2.6 Schematic illustration of main transitions and reactions directly involved with photodynamic reactions. *Arrows* in *squares* represent electron spin configurations in valence shell orbitals of radicals and singlet- and triplet-state molecules. *ISC* intersystem crossing

energy configurations and may assume distinct positions in relation to the nucleus due to altered repulsion to surrounding electrons, as represented by the horizontal axis in Fig. 2.5.

The definition of free radicals or singlet (S) and triplet (T) states can be based on the presence and spin of electrons in orbitals of the valence shell. As illustrated in Fig. 2.6, singlet states have paired electrons with opposite spins (indicated by arrow direction), triplet states have two unpaired electrons with same spin, and radicals have one unpaired electron. Therefore, when an excited singlet state (S*) decays to a triplet state, the electron spin is inverted (flipped). Since excitation to a triplet state involves an additional (forbidden) spin transition, it is less probable that a triplet state will form when the molecule absorbs radiation. The nonradiative spin-forbidden transitions (e.g., S→T or T→S) are named intersystem crossing (ISC). It normally is very fast (<10 ns) among higher excited states (e.g., S*→T*), but may take from microseconds up to days to decay from the lowest triplet excited state to a singlet ground state (e.g., T*→S_0). The long-lived triplet state is a key point to provide enough time (above microsecond scale) for molecular interactions and allow chemical reactions to occur. By comparison the lifetime of the excited singlet state is measured in a few nanoseconds or less, which is insufficient to allow chemical reactions to occur.

Internal conversion (IC) transitions are also nonradiative but differ from intersystem crossing because electron spin remains the same (i.e., spin-allowed transition). The spin-allowed nonradiative transitions, as in IC or within vibrational modes of a particular electronic state, are exceedingly fast (usually in subnanosecond scale). Radiative transitions without spin inversion (e.g., S*→S or T*→T) are usually very fast events (<10 ns) typified by the lifetime of fluorescent photon emission. As said before, the triplet transition to the ground-state singlet takes longer to occur, and the lifetime of phosphorescence emission is generally more than 1,000 times longer than fluorescence. It is important to remark that since nonradiative transitions may dissipate some of the energy initially absorbed, photons emitted by fluorescence or phosphorescence have lower energy (longer wavelength) than the absorbed photon.

Excited molecules can alternatively be deactivated, or quenched, by donating charges or energy to a nearby ground-state molecule. Because of spin and symmetry

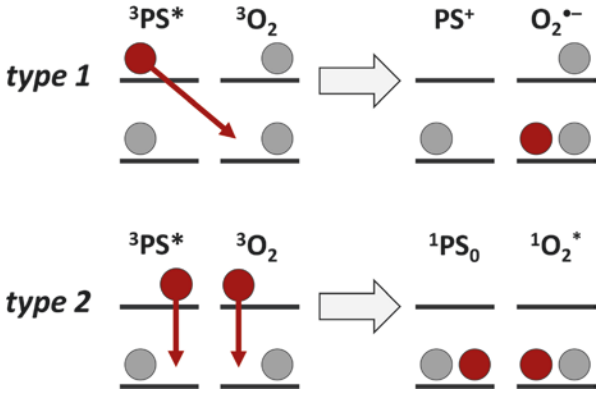

Fig. 2.7 Illustration of electronic transitions of photodynamic reactions. In type 1 reactions, excited triplet-state PS ($^3PS^*$) donates one electron to triplet oxygen ($^3O_2$) to produce superoxide radical anion ($O_2^{\bullet-}$). Type 2 reactions produce singlet oxygen ($^1O_2^*$) via energy donation from $^3PS^*$ to $^3O_2$ through a coulombic mechanism of electronic transition, also named as Förster or static quenching mechanism. Differently from type 1 mechanism, a dipole-dipole interaction can occur without the requirement of collision between donor and acceptor molecules and can occur in distances up to 5 nm

selection rules, i.e., triplet-triplet interactions are spin-allowed while triplet-singlet interactions are spin-forbidden, the excited triplet PS may preferentially interact with other molecules also in triplet configuration. Curiously, the vast majority of molecules found in nature are singlets in the ground state (S_0). There only a very few exceptions that are stable as triplets in their ground state, and the only one of any practical significance is molecular dioxygen ($\mathbf{O_2}$). Also, ground-state O_2 is a diradical molecule by definition, i.e., it has two electrons alone occupying two different orbitals in the same molecule (Fig. 2.7). Being stable as a diradical triplet allows O_2 to chemically or physically interact with excited triplet molecules (e.g., photosensitizers) either via one or two electron(s) transitions. Therefore, molecular interactions between excited-state PS and ground-state O_2 is a remarkably favored process. Photodynamic reactions rely principally on such intermolecular events.

When an excited triplet-state PS ($^3PS^*$) meets triplet ground-state oxygen ($^3O_2$), two main reaction types may occur: *type 1*, one electron transfer to oxygen generating "superoxide radical anion" ($O_2^{\bullet-}$) and the $PS^{\bullet+}$ radical cation, or *type 2*, energy donation to oxygen generating excited state "singlet oxygen" ($^1O_2^*$) and ground-state PS. As a matter of fact, other secondary photochemical reactions may have some influence upon photodynamic therapy effects, depending on the photosensitizer employed. The excited PS may also donate H^+ ions to the solvent, be cleaved to become a toxic ground-state compound, or even directly interact with organic substrates to induce their release of free radicals. However, using specific chemical inhibitors (e.g., superoxide dismutase, sodium azide, dimethylthiourea, mannitol, etc.), it was demonstrated that for the best performing photosensitizers, these are only side reactions that have a minor influence upon cell killing caused by PDT [5]. Therefore, we will focus on quenching mechanisms through charge and especially energy donation, mediated by O_2, as illustrated by Figs. 2.6 and 2.7.

In type 1 reactions, ^{3}PS* donates one electron to ground-state O_2 and yield $O_2^{\bullet-}$ (Figs. 2.6 and 2.7). This product is mildly reactive with biomolecules but can be reduced to hydrogen peroxide (H_2O_2), a non-radical and less reactive specie. In biological systems this reaction is constitutively catalyzed by enzymes from the superoxide dismutase (SOD) family, which occur broadly in nature [6, 7]. Hydrogen peroxide can be further reduced through reactions catalyzed by catalase (CAT) enzymes and produce $H_2O + O_2$. On the other hand, H_2O_2 may gain a second electron by transfer from the excited-state triplet PS to give the most reactive oxygen species: the hydroxyl radical (OH•). This one-electron reduction may also occur via the intermediacy of transition metal ions such as Cu^+ or Fe^{2+} (Fenton reaction).

Singlet oxygen ($^1O_2$*) is formed as product of type 2 reactions. Even though referred as secondary reactions due to its later discovery, $^1O_2$* is the main ROS produced by most commercially available PS. The exact singlet oxygen production efficiency and yield is directly dependent to the photosensitizer structure, the local oxygen concentration, and which solvent they are diluted in [8, 9]. To produce singlet oxygen, the energy donation from ^{3}PS* to $^3O_2$ takes place through a coulombic mechanism of electronic transition, also named as Förster or static quenching mechanism. In this event, ^{3}PS* donates its deactivation energy to $^3O_2$ producing $^1O_2$* and $^1PS_0$ (Figs. 2.6 and 2.7). The electric dipole transition nature of coulombic (or Förster) mechanism allows ^{3}PS* to efficiently induce the formation of $^1O_2$* at distances over 5 nm [10]. Due to its generally high redox potential, $^1O_2$* is a powerful oxygenating agent inducing cycloaddition reactions, but may also be reduced to $O_2^{\bullet-}$ and produce typically type 1 ROS (Fig. 2.6). Conversely $O_2^{\bullet-}$ can sometimes act as a reducing agent by donating the unpaired electron to a substrate, and when this happens the product of this reaction is $^1O_2$* (not $^3O_2$) [10]. Therefore, type 1 and type 2 pathways are not "set in stone" and crossover between different ROS can occur. The main factor influencing this crossover is the redox potential of the PS (in its ground state and its triplet state) and the redox microenvironment of the PDT reaction. Since $^1O_2$* is an excited-state molecule, it can also spontaneously decay to ground state via phosphorescence. The lifetime of $^1O_2$* in pure water is on the order of 3 μs; this permits a diffusion radius of nearly 100 nm. In the intracellular environment, the diffusion radius is much reduced because there are several targets that can react with $^1O_2$* [11]. Therefore, in cells $^1O_2$* mostly reacts with biomolecules present in the surrounding of its formation, highlighting the importance of the site of accumulation of the PS. As we will discuss in Chap. 4, $^1O_2$* has a ubiquitous reaction pattern with biomolecules such as aromatic and sulfur-containing amino acids, unsaturated lipids, steroids, and nucleotides [12]. Intense molecular damage caused by $^1O_2$* can lead to cell death via apoptosis, autophagy, or necrosis.

Depending on their electronic configuration and redox potential, each photosensitizer molecule may preferentially undergo either a type 1 or type 2 reaction, although both reaction pathways usually compete along with self-deactivation processes (Fig. 2.4). Photosensitizers that preferentially undergo type 1 photodynamic reactions are more susceptible to cellular antioxidant defense since there are specific detoxifying enzymes for ROS such as superoxide and hydrogen peroxide. Constitutive overexpression of superoxide dismutase, catalase, peroxiredoxin, and

glutathiones or accumulation of manganese ions can represent effective protection against oxidation by hydrogen peroxide or superoxide and hydroxyl radicals. All these mentioned features can be sufficient to impose challenges for PDT to treat tumors and microbial strains resistant to traditional chemotherapy and radiotherapy.

Differently from type 1 ROS, there are no enzymes that are able to efficiently inactivate $^1O_2$*, and nearly all cellular defenses rely on finite stocks of scavenger and quencher molecules that quench ROS by directly interacting with them (see Chap. 4 and 5). Since during PDT each PS molecule can produce more than 10,000 singlet oxygen molecules per second before it is destroyed by photobleaching, photosensitized cells are simply not equipped with enough antioxidant capacity to tolerate such intense attack for longer than a few minutes. In fact, there are only a few possible tolerance or resistance mechanisms to PDT ever reported, such as production of melanin granules and sequestration of PS inside them, or pumping the PS out of the cell by multidrug efflux pumps. These mechanisms of tolerance, and how to overcome them, will be posteriorly discussed in Chap. 5.

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