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Aust. J. Chem. **2008**, *61*, 741–754

Photodynamic Therapy: Porphyrins and Phthalocyanines as Photosensitizers

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The present work is focussed on the principles of photodynamic therapy (PDT), emphasizing the photochemical mechanisms of reactive oxygen species formation and the consequent biochemical processes generated by the action of reactive oxygen species on various biological macromolecules and organelles. This paper also presents some of the most used photosensitizers, including Photofrin, and the new prototypes of photosensitizers, analysing their physicochemical and spectroscopic properties. At this point, the review discusses the therapeutic window of absorption of specific wavelengths involving first- and second-generation photosensitizers, as well as the principal light sources used in PDT. Additionally, the aggregation process, which consists in a phenomenon common to several photosensitizers, is studied. J-aggregates and H-aggregates are discussed, along with their spectroscopic effects. Most photosensitizers have a significant hydrophobic character; thus, the study of the types of aggregation in aqueous solvent is very relevant. Important aspects of the coordination chemistry of metalloporphyrins and metallophthalocyanines used as photosensitizers are also discussed. The state-of-the-art in PDT is evaluated, discussing recent articles in this area. Furthermore, macrocyclic photosensitizers, such as porphyrins and phthalocyanines, are specifically described. The present review is an important contribution, because PDT is one of the most auspicious advances in the therapy against cancer and other non-malignant diseases.

Manuscript received: 10 April 2008.
Final version: 24 July 2008.

Photodynamic Therapy

Introduction

Photodynamic therapy (PDT) is a promising therapeutic treatment for various tumours and non-malignant diseases. The technique requires the presence of a dye, preferably located in the target tissue to be treated, a light source, and molecular oxygen. Although many sensitizer molecules can naturally occur as constituents of cells and tissues, in PDT they must be introduced into the organism in a first step. In a second step, the tissue-localized sensitizer is exposed to light of adequate wavelength, leading to a sequence of biological processes through different photophysical pathways. The mechanism involves the production of reactive oxygen species (ROS), which results in cell death.[1] This method has been employed as therapy against different tumour types, such as skin, bladder, oral cavity, and others.[2] In fact, PDT is an US Food and Drug Administration-approved modality that rapidly eliminates local tumours, resulting in cure of early disease and palliation of advanced cases, being associated with an enhanced antitumour immunity.[3] Likewise, PDT has advantages over other cancer treatment modalities, such as surgery, radiotherapy, and chemotherapy. Owing to its potential to selectively target malignant cells, the technique decreases the damage to healthy tissues.[4]

Despite PDT being originally developed as a tumour therapy, it has been successfully employed in the treatment of non-malignant disease.[5–7] Among those applications, it is interesting to mention the potential of this technique as antimicrobial therapy. This treatment may be a valuable tool to achieve a rapid reduction of the microbial burden, and perhaps even in the management of localized infections that are resistant to standard antibiotics regimens. Several photosensitizers have been effective in the photokilling of Gram-positive and Gram-negative bacterial pathogens, as well as parasites, fungi, and viruses.[8–10] Actually, PDT has been applied against leishmaniasis, in which *Leishmania major* promastigotes are photo-inactivated by PDT employing tetracationic porphyrins as photosensitizer.[11] Cutaneous leishmaniasis caused by *Leishmania major* has also been treated with topical PDT.[12] PDT has also demonstrated potential to generate cell death in *Tritrichomonas foetus*, which causes bovine trichomoniasis.[13]

PDT has been known for over a 100 years, but it has only now been widely used.[5–7] In fact, the association of photodynamic action with therapeutic results has been know since the time of the ancient Egyptians.[14] The scientific basis for such use was vague or non-existent before ~1900.[15] The 1903 Nobel Prize was awarded to Niels Finsen for his work on phototherapy. Finsen discovered that light treatment could control skin manifestations of tuberculosis, a very common ailment at that time.[16]

PDT has been employed with success in various countries, such as Brazil, where, since 1997, a collaborative program has treated over 400 cancer patients (by late 2004). The photosensitizers used in this program are those approved for

 10.1071/CH08145 0004-9425/08/100741

use in humans by the US FDA, such as, for example, ALA (5-aminolevulinic acid). About 80% of lesions were located in the head and neck or skin. However, this Brazilian program has also obtained excellent results for the treatment of other kinds of malignancies.[17]

Reactive Oxygen Species

Oxidative stress has been defined as a disturbance in the pro-oxidant–antioxidant balance, in favour of the former, leading to potential damage. This imbalance may be due to an increased production of various reactive species and a decreased ability of the natural protective mechanisms of the organism to inhibit the action of these reactive compounds. Injury to cells occurs only when the reactive ROS overwhelm the biochemical defences of the cell.[18]

Potentially cytotoxic ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^-) can be generated in eukaryotic cells during the course of normal energy metabolism (mitochondrial electron transport and oxidative phosphorylation) or metabolism of redox-cycling xenobiotics, such as anthracyclines.[19] Indeed, even the exposure to natural sunlight may be an important source of ROS in humans as a result of the excitation of the endogenous photosensitizers in the skin.[20]

Furthermore, ROS can also be generated by numerous extracellular agents, such as ionizing and non-ionizing radiation, for example. Special interest has been dedicated to this last category. Radiation near ultraviolet (UVA, 320–400 nm) and visible (400–700 nm) light, used with appropriate photoexcitable compounds (sensitizers) and ground-state molecular oxygen ($^3O_2$), produce oxidative injury through photodynamic action.[19]

Singlet molecular oxygen is a highly reactive form of oxygen (ROS) that reacts with many biological molecules, including lipids, proteins, and nucleic acids.[21] This action is associated with different cell organelles, such as plasma membrane, nuclei, mitochondria, Golgi apparatus, lysosomes,

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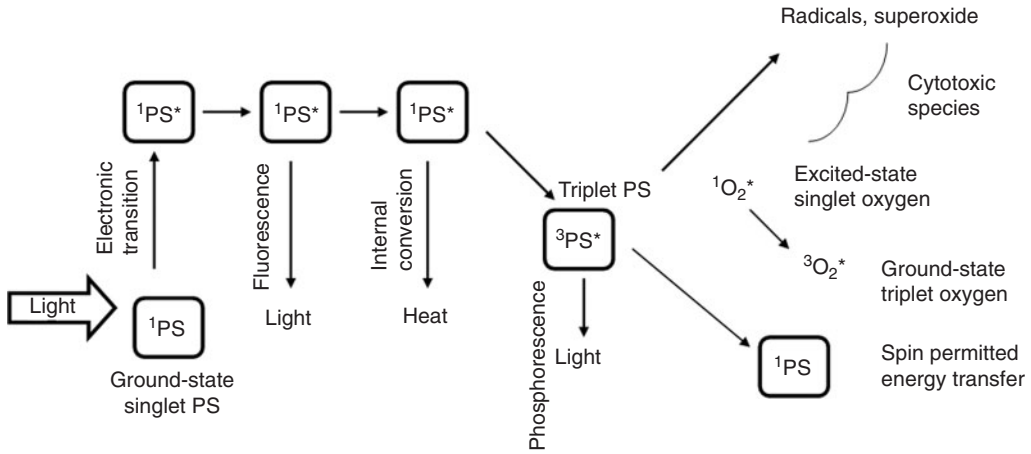

Fig. 1. Schematic representation of the photochemical process of excitation of the photosensitizer (PS), including the possibilities of luminescence (fluorescence and phosphorescence), the singlet and triplet excited states and the reactive oxygen species generated by the energy transfer from the PS.

and cytoskeletal structure, which are the most susceptible to photo-induced oxidative stress. Free radicals and other reactive species are involved in different human diseases such as rheumatoid arthritis, Alzheimer's, Parkinson's, carcinogenesis, cardiovascular diseases, AIDS, and cataract formation, as well as in non-pathological processes, such as aging.[18]

In fact, singlet oxygen is the putative cytotoxic agent in PDT, initially formed by the transfer of energy from an excited photosensitizer to ground-state oxygen molecules.[22,23] Two basic types of reaction can occur following photosensitizer photoactivation. One mechanism involves free-radical generation and tumoural destruction through direct oxidation of cell structures (type I photochemical reaction). The other mechanism provokes the singlet oxygen production through energy transference from excited triplet species to molecular oxygen species, which mediates oxidation processes (type II photochemical reaction)[24] (Fig. 1).

The type II mechanism is commonly considered as the main one responsible for cell damage. Quenching of π–π^* excited triplet states by O_2 proceeds via internal conversion of excited encounter complexes and exciplexes of sensitizer and O_2. Both deactivation channels lead with different efficiencies to singlet oxygen generation. The balance between the deactivation channels depends on the triplet-state energy, the oxidation potential of the sensitizer and the solvent polarity.[25] In any case, the quenching of the lowest electronically excited states of most substances by O_2 produces electronically excited oxygen. Both the lowest excited singlet states of O_2 are formed if the energy transferred exceeds the excitation energy E_Σ 157 kJ mol^{-1} of the upper excited singlet oxygen species O_2.[25]

In Eqn 1, it can be observed that the electron transfer from RH (chemical compound present acting as reductant) to ^{3}PS (photosensitizer in its triplet state) results in the formation of two free radicals, $PS^{-\bullet}$ (sensitizer radical anion) and $R^\bullet$ (substrate radical). Both radicals could induce a chain peroxidation cascade in the biological medium. Reduced oxygen species (O_2^-, H_2O_2, $HO^\bullet$) are commonly generated by hydrogen or electron transfer from a biological reductant (RH) (for example, NAD(P)H, ascorbate, glutathione) to ^{3}PS (type I process), followed by autoxidation of the latter.[19]

$$^3PS + RH \rightarrow PS^{-\bullet} + R^\bullet + H^+ \qquad (1)$$

Among the excited species, singlet oxygen ($^1O_2$) is of particular interest because of its high electrophilicity and different lifetimes depending on the solvent and medium conditions (for example, 2–4 μs in aqueous solvent and ~700 μs in CCl_4). This excited species exhibits high reactivity and can be produced in biological systems. In mammalian cells, singlet oxygen species can be generated during oxidative stress and they are able to attack DNA, protein thiol groups, and membrane lipids.[26] It is important to notice that singlet oxygen is so highly reactive that its interaction with cell membranes may generate secondary products that could react with DNA, leading to mutagenic lesions in a kind of synergic effect of the initial reaction.[26]

Indeed, DNA and proteins are important targets for the prooxidant action of singlet oxygen species.[27,28] This action on DNA has been reported to occur in PDT, and this damage may be an important factor in the success of this therapeutic modality.[29] Considering haemeproteins, for instance, this excited species can damage both apoproteins and prosthetic groups. Because the vital biological function of these proteins is dependent on the integrity of the haem group, a particular resistance to modifications by reactive species is expected in the haem group. In fact, when haemoglobin and *c*-phycocyanin are exposed to this species, chemical alterations of the chromophore constitute only a minor reaction pathway. However, although data reported in the literature have demonstrated that singlet oxygen induces modifications in the haem group of *Neurospora crassa* catalase, the chemical nature of the modifications is still under investigation. In the apoprotein structures, amino acids are among the main sites of attack by oxygen singlet species.[24]

It is important to register that, independently of the ROS action, accumulating evidence indicates that antitumour effects are also mediated by indirect stimulation of inflammatory and immune responses, which include rapid local infiltration of tumours by neutrophils and macrophages accompanied by systemic release of inflammatory mediators.[30] This immune response is usually associated with a 'low-dose PDT' that occurs when one of the three main PDT agents (photosensitizer, ground-state molecular oxygen, and light intensity) are found at low levels in the vicinity of the tumour.[31] This early response can became a more precise immune reaction that involves activation of specific T lymphocytes that seem to be necessary for the ultimate control of residual tumour cells.[30] Moreover, it has been shown that low-power laser light intensity is immunostimulative

and this action is associated with primary chromophores, such as endogenous porphyrins, through a photodynamic process.[32] Indeed, several papers have argued that the inflammation process provoked by photodynamic action is associated with a protective response that initiates several processes of tissue repair through ROS.[33]

The singlet oxygen quantum yields (Φ_Δ) are strongly dependent on the π-conjugated system of the photosensitizers, justifying the widespread employment of π-conjugated macrocycles, such as porphyrins and phthalocyanines. The relationship between the molecular structure and the singlet oxygen quantum yields (Φ_Δ) can be associated with the relationship that occurs between the intersystem crossing rate constant ($S_1 \rightarrow T_1$) and the energy difference between S_1 and T_1 (S_1 corresponds to the lowest excited singlet state and T_1 is the lowest excited triplet state). The singlet oxygen quantum yields could be controlled by changing the symmetry of π-conjugated systems, which has been studied to promote the preparation of novel photosensitizers.[34]

Sensitizer Targeting and Absorption

The reduction of the size of damage site in order to avoid any action on normal cells is one of the main scopes of recent studies about PDT. Many photoactivable molecules have been synthesized, such as porphyrins, chlorins, and more recently phthalocyanines, which present strong light absorption at wavelengths around 670 nm and are therefore well adapted to the optical window required for PDT application. Actually, for employment in vivo, photosensitizer requires a maximum of optical absorption between 650 and 780 nm to avoid the parallel absorption by the endogenous dyes, such as haemoglobin. This molecule competes with the exogenous photosensitizer for the photons that are irradiated in the tissue. However, the lack of selective accumulation of these photoactivable molecules within tumour tissue is a major problem in PDT, and an important research area is the development of targeted photosensitizers. An interesting point recently is the association of photosensitizers and biomolecules, such as synthetic peptides.[35,36] In this context, an attempt is the conjugation of photosensitizers, such as phthalocyanines, with biomolecules that possess a marked selectivity towards cancer cells. This association would improve subcellular localization, delivering the dye to photosensitive sites within the cells.[35,37] It is important to note that a significant number of sensitizers employed in experimental or clinical PDT act in the plasma membrane, mitochondria, endoplasmic reticulum, and lysosomes.[38–40]

There is increasing evidence that the solubility of the photosensitizer, i.e. its uptake and retention in membranes, is critical, if not a fundamental prerequisite, for the drug activity and efficacy. Although pigment solubility in membranes seems to correlate with both membrane and drug (for example, porphyrins and porphyrinoids) characteristics, the structure–solubility and structure–activity relationships remain topics of great controversy.[41] Indeed, to characterize structure–solubility and structure–function relationships, systematic studies of the interaction between the drugs and the membranes are necessary to determine results for the subsequent topics: (i) the level of correlation between membrane incorporation and structural molecular and electronic properties of the photosensitizer; (ii) the mechanism of interaction involving the photosensitizer and membranes; and (iii) the location in the membrane or in the membrane–water interface where the photosensitizer is positioned.[41]

Another important point to be mentioned is that the radius of action of the ROS is lower than 0.02 μm. This small radius makes the damage site restricted to the area in which ROS are produced. In this way, an important advantage of PDT is that it provides a selective therapeutic effect, sparing surrounding normal tissue by preferential accumulation of the photosensitizer in tumour tissue and laser irradiation restricted to the target tissue.[42]

Cell Death

PDT may cause cell death by necrosis or apoptosis, both in vitro and in vivo.[21] The mechanism of tissue damage from PDT may be cellular, vascular or both, depending on the photosensitizing agent and the treatment conditions. Changes in the vasculature may include vascular stasis, vascular leakage, or vessel collapse leading to ischaemic necrosis.[43]

Efficient cell death is observed when light, oxygen, and photosensitizer are not limited ('high-dose PDT'). When one of these components is limited ('low-dose PDT'), most of the cells do not immediately undergo apoptosis or necrosis but are growth-arrested with several activated transduction pathways.[31]

Necrosis has been considered a kind of accidental cell death caused by physical or chemical damage through a non-programmed process.[6,44] The necrotic cell death is characterized by cell and organelle swelling, leading to the disruption of cell membrane and cell lysis,[45] thus leading to the release of intracellular contents and inflammation.

Apoptosis is a genetically controlled cell 'suicide' pathway that is utilized to eliminate unwanted or potentially harmful cells under a variety of physiological and pathological situations in multicellular organisms. This process has an important role for normal development, tissue homeostasis, and the action of cytotoxic anticancer drugs.[46,47] Morphologically, apoptotic cell death is characterized by plasma membrane blebbing, cell shrinkage, condensation of the nucleus, degradation of proteins and DNA, and formation of the apoptotic bodies.[45,48] Apoptosis limits leakage of intracellular material to the immediate environment and, consequently, avoids tissue inflammation.

Cell death is directly related to some groups of proteins that are responsible for the activation and execution of the events observed in apoptosis. Caspases (Cysteine ASPartate-specific proteaASEs) are a family of proteases that are constitutively present in most mammalian cells. These proteases reside in the cytosol as single-chain proenzymes.[49] In apoptosis, caspases function in both cell disassembly (effectors) and in initiating this disassembly in response to pro-apoptotic signals (initiators).[50] The caspases act as effectors when they cleave and inactivate proteins that protect living cells from apoptosis, such as DNA repair proteins, the ICAD (Inhibitor of Caspase-Activated Deoxyribonuclease – responsible for the DNA degradation in apoptosis) or Bcl-2 family proteins, among others.[21,51]

The apoptotic process can be activated by internal signalling (intrinsic pathway) or by external signalling (extrinsic pathway). The intrinsic pathway relies on mitochondrial membrane permeabilization (MMP) to liberate the electron transport chain intermediate cytochrome *c* from the mitochondrial intermembrane space. Once released to the cytosol, cytochrome *c* can combine with deoxy-adenosine triphosphate (dATP), apoptotic protease activating factor 1 (APAF-1), and caspase-9 to form the apoptosome. This catalytic complex is responsible for the activation of effectors caspase-3 and -7.[49] The extrinsic pathway is initiated by the interaction of cytokines of the tumour necrosis factor (TNF) family with specific receptors present at the plasma membrane. This interaction promotes the recruitment of adaptor

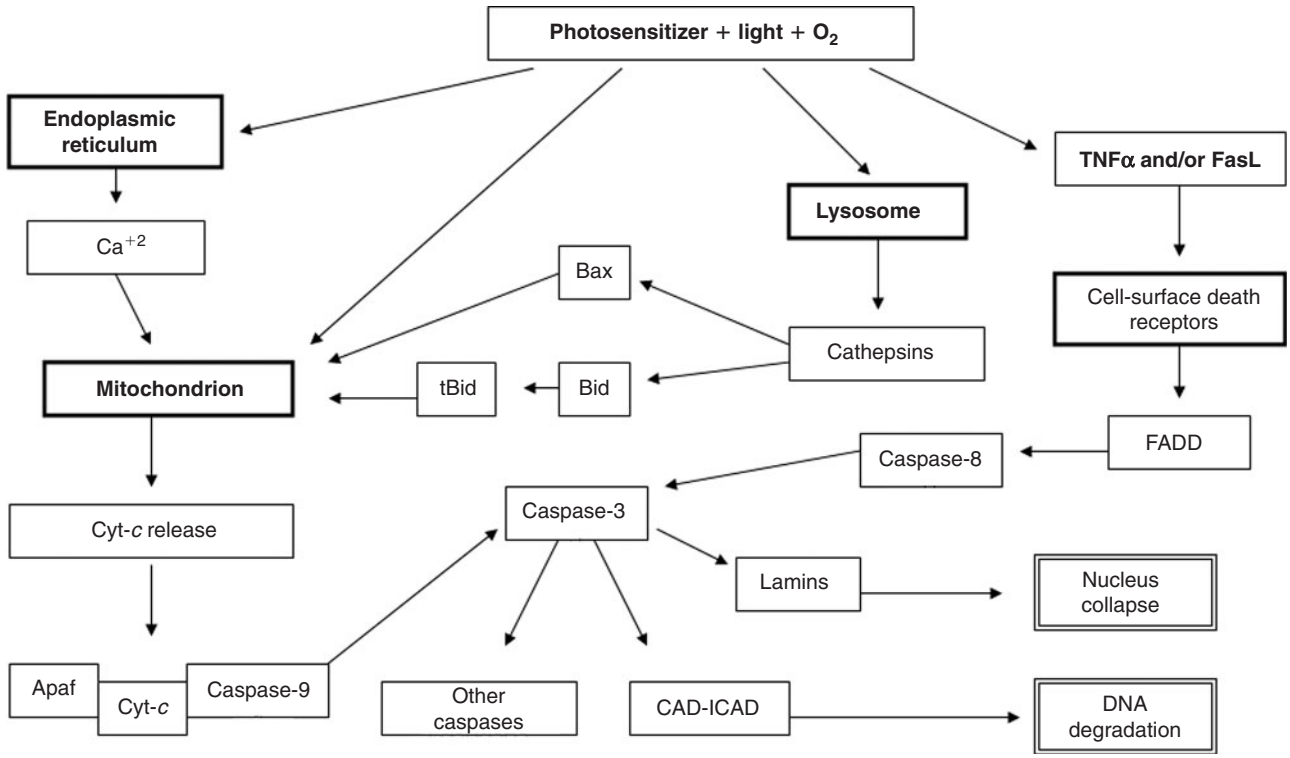

Fig. 2. Some possible apoptotic pathways unleashed by photodynamic therapy (PDT). The effects are dependent on the localization of the photosensitizer. The PDT can induce damage to the mitochondria membrane, with consequent release of cytochrome *c* (Cyt-*c*). In cytoplasm, the Cyt-*c* interacts with the proteins Apaf and caspase-9, forming the so-called apoptosome. This complex activates caspase-3, which activates other effector caspases and directly cleaves several substrates, such as lamins, structural and adhesion proteins promoting the degradation of the nucleus and other cell structures. Furthermore, caspase-3 cleaves inhibitor of caspase-activated deoxyribonuclease, liberating CAD protein that degrades DNA. PDT can induce damage in the endoplasmic reticulum, promoting liberation of Ca^{+2}, and this ion can cause mitochondrial alterations and Cyt-*c* release. If the photosensitizer interacts with the lysosome, cathepsin can be released. This can result in the cleavage of Bid, resulting in tBid, which promotes destabilization of the mitochondrial outer membrane and liberation of Cyt-*c*. Other possible effects of the cathepsin correspond to the direct or indirect activation of Bax protein, another pro-apoptotic Bcl-2 family member. PDT induces liberation of cytokines of the tumour necrosis factor (TNF) family. The interaction of these cytokines with TNF-R1 and Fas cell-surface receptors activates the initiator caspase-8, which cleaves Bid protein or directly activates caspase-3.

proteins responsible for the activation of initiator caspases, such as caspase-8 and caspase-10.

Another important protein family that has a central role to the apoptotic process is the Bcl-2, which presents pro-apoptotic (e.g. Bax, Bad) and anti-apoptotic (e.g. Bcl-2, Bcl-XL) elements. During conditions of cell stress, anti-apoptotic Bcl-2 family members present at the outer mitochondrial membrane can be destabilized through decreased expression, or by the induction of pro-apoptotic Bcl-2 family members. In the latter situation, the ratio of pro-apoptotic family members to anti-apoptotic family members becomes greater, allowing the formation of proteinaceous outer membrane channels by the pro-apoptotic Bcl-2 family members, which can liberate cytochrome *c*.[52,53]

It was originally proposed that the initiation of apoptosis by PDT derived from direct mitochondrial photodamage.[54] However, earlier studies showed that Bcl-2 was the initial target of many photosensitizing agents.[55,56] Photodamage to Bcl-2 and related proteins results in the release of pro-apoptotic molecules and the subsequent initiation of apoptosis.[57] Bcl-2 and other anti-apoptotic proteins can occur both at the endoplasmic reticulum and mitochondrial sites and photodamage at either place can have pro-apoptotic consequences.[58]

Lysosomal proteases have been associated with the initiation of apoptosis. Several investigators have reported that the cytokine TNFα stimulates the release and translocation of lysosomal proteases into the cytosol, which could lead to the release of cytochrome *c* and activation of the intrinsic apoptotic pathway.[59] The photosensitizer mono-L-aspartyl chlorin e6 (NPe6) localizes on lysosomes.[60] Irradiation of cultures preloaded with NPe6 induced the rapid destruction of lysosomes, and subsequent cleavage and activation of Bid (a pro-apoptotic Bcl-2 family member) and pro-caspases-9 and -3.

The local photodamage to specific subcellular targets critically influences the kinetics and the regulatory pathways activated, as well as the mode of cell death, e.g. apoptosis or necrosis, following PDT.[61] Fig. 2 illustrates some apoptotic pathways induced by PDT. According to Agostinis and coworkers,[61] sensitizers with preferential mitochondrial localization or located in other organelle membranes will promote apoptosis, whereas compounds localized in the plasma membrane will induce a necrotic process. The intracellular localization of the photosensitizer coincides with the primary site of the photodamage, mainly because of the limited diffusion of the short-lived singlet oxygen that is thought to be the predominant oxidant in PDT.[21] The incubation time of cells with photosensitizer is another parameter that affects the mode of cell death during exposure to light. The plasma membrane is a very important site of damage when short periods of incubation are used. However, Photofrin, as well as phthalocyanines, tend to localize in the mitochondrial membrane after long periods of incubation.[40,62,63]

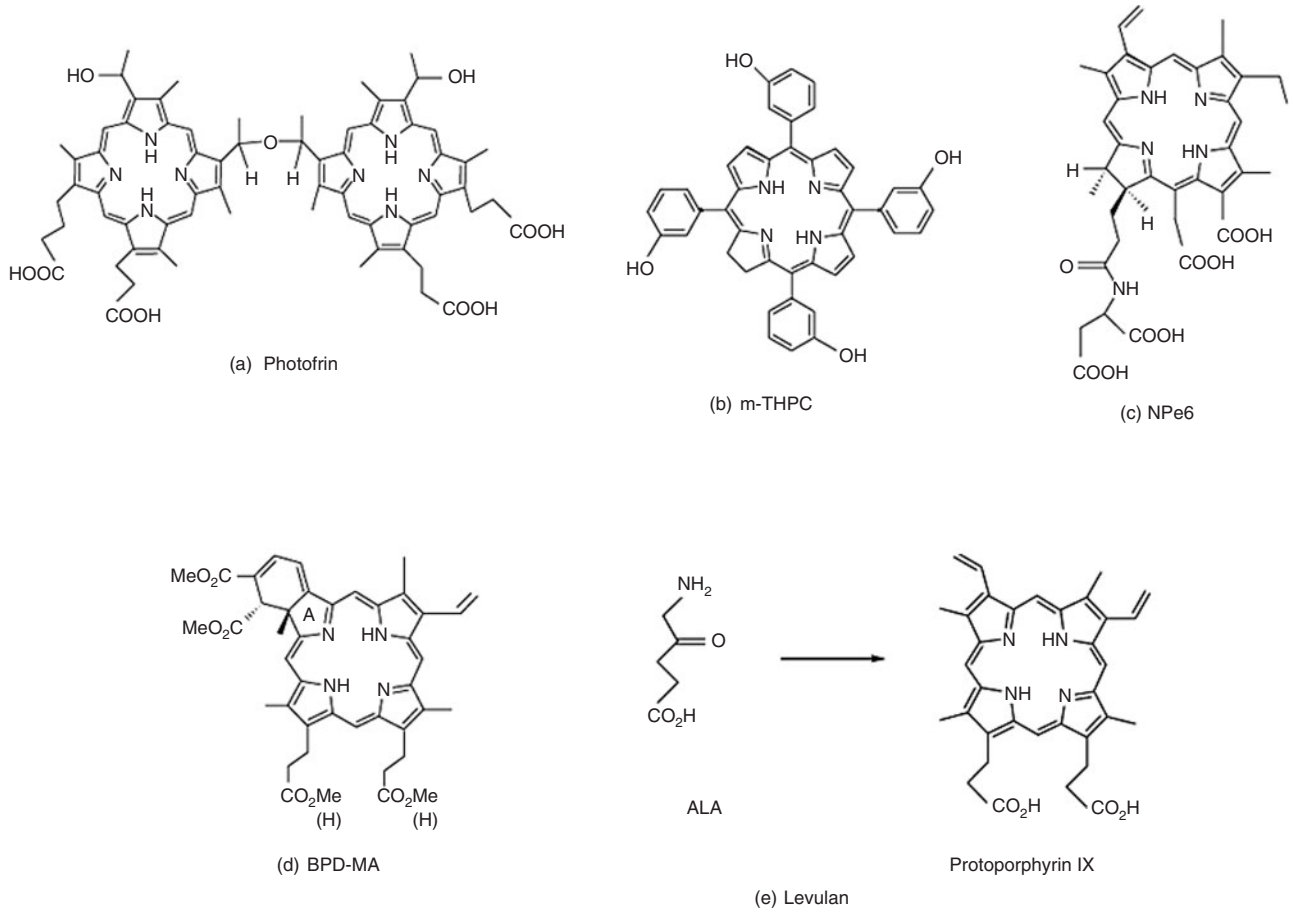

Fig. 3. Structural representation of the most common photosensitizers employed in photodynamic therapy.

Photosensitizers Employed in PDT

In 1912, Meyer-Betz showed that haematoporphyrin (Hp), a compound isolated from haemoglobin, was an extremely powerful photosensitizer with tumour-localizing properties.[64] The purification of Hp led to the preparation of haematoporphyrin derivative (HpD), a complex mixture of components with better tumour localization than crude Hp.[65,66] HpD was further purified by Dougherty,[67] which allowed the preparation of porfimer sodium (Photofrin). HpD and Photofrin were the first photosensitizers to be systematically studied for clinical PDT.[29,68]

Photofrin is not a chemically defined entity, but rather a mixture of porphyrins, monomers, dimmers, and oligomers that contain impurities[29,42] (Fig. 3a). This photosensitizer is employed in the treatment of a great number of solid tumours, including cancers of the lung, stomach, oesophagus, and uterine cervix.[42] The major drawback to the use of Photofrin is the fact that it causes prolonged skin photosensitization, lasting up to 4–6 weeks after treatment. Besides, its activating wavelength (630 nm) corresponds to a low-absorption photosensitizer profile, exhibiting poor tissue penetration. This point must be emphasized because one of the characteristics of the 'ideal' photosensitizer for in vivo application is a maximum light absorption in the red region of the visible spectrum (650–780 nm). This characteristic is desirable in order to avoid the absorption of the light due to the presence of endogenous pigments, mainly haemoglobin, which strongly absorbs light between 400 and 600 nm.[42] However, it is important to note that the energy of singlet oxygen O_2 ($^1\Delta_g$) is $\sim$94 kJ mol^{-1}, which corresponds to the energy of 1270 nm. However, wavelengths longer than 800 nm are rarely used for PDT owing to high scattering in tissue and lack of adequate sensitizers that absorb in this region of the spectrum.

The considerations mentioned above prompted an active search for novel, chemically well-defined photosensitizers with improved biological properties, including better optical and pharmacokinetic characteristics, generally known as 'second-generation photosensitizers'.[29,42]

Most of the second-generation photosensitizers are porphyrin-like molecules, such as benzoporphyrins, chlorins, pheophorbides, texaphyrins, phthalocyanines, and naphthalocyanines. Second-generation photosensitizers have some of the following characteristics: (i) high absorption coefficients and quantum yields; (ii) depth penetration in mammalian tissues due to their wavebands absorption (660–700 nm far red and 700–850 nm near infrared); (iii) short serum half life and selective tissue accumulation; (iv) minimal toxicity in the absence of light; and (v) they correspond to pure compounds.[68]

Meta-tetra(hydroxyphenyl) chlorin (mTHPC or Foscan) (Fig. 3b) is a second-generation photosensitizer that mediates cell photodamage through singlet oxygen formation.[69] Fluorescence microscopy studies in monoculture cells showed intense fluorescence in the perinuclear region, endoplasmic reticulum, and Golgi apparatus.[70,71] This photosensitizer has been approved in Europe for treatment of head and neck cancers.[69]

NPe6 is a hydrophilic chlorin derived from chlorophyll with excellent photosensitizing properties in vitro and in vivo[72,73] (Fig. 3c). NPe6 is chemically pure and demonstrates significant absorption at 664 nm. The compound tends to localize in the lysosomal compartment of cells and has in vivo tissue distribution properties similar to Photofrin.[73]

Benzoporphyrin derivative monoacid ring A (BPD-MA, Verteporfirin) (Fig. 3d) is a second-generation porphyrin photosensitizer derived from protoporphyrin.[68] This agent has a rapid uptake, fast clearance, and is selectively taken up by endothelial cells.[74,75] BPD-MA has been used for the treatment of age-related macular degeneration.[31]

Some of the currently available photosensitizers are highly hydrophobic compounds, often with long retention times (i.e. weeks) in tissues and difficult to formulate. This difficulty has recently motivated several strategies to improve the solubility and tumour-specificity of PDT photosensitizers, including their conjugation to carrier proteins, oligonucleotides, monoclonal antibodies, epidermal growth factors, carbohydrates, and hydrophilic polymers.[4]

In this way, the synthesis and evaluation of new photosensitizers, such as porphyrins with cationic substituents groups, continue to be one of the main goals in the area of PDT.[76,77] At present, it is known that cellular uptake and targeting of subcellular organelles occur more efficiently with lipophilic photosensitizers, owing to their better diffusion through cell membranes.

The earlier metabolic precursor, ALA (Levulan), is not a photosensitizer by itself but is metabolized to photosensitive protoporphyrin IX (Fig. 3e) through the haem biosynthetic pathway.[68] The rate of synthesis and accumulation of ALA-induced protoporphyrin IX is higher in malignant and premalignant cells than in normal cells. Furthermore, this compound shows low toxicity and is rapidly cleared from the body by the existing clearance mechanism. In addition, protoporphyrin IX induced after ALA application produces a fluorescence that can be visualized under blue light. In this way, ALA can be employed in diagnosis.[64]

Light Sources Employed in PDT

Lasers, as well as non-coherent light sources, have been used for PDT. The advantage of using lasers is that they can be coupled into fibre optic systems and led to otherwise inaccessible locations, such as urinary bladder, digestive tract, or brain. For dermatology, however, non-lasers sources are superior to laser systems because of their large illumination field, lower cost, smaller size, reliability, and easy setup.[78,79] In fact, together with the scarce knowledge of the benefits of PDT by physicians, the cost of the light source is one of the main factors that limits the wide employment of this therapeutic methodology in several countries.[80] The choice of the light source depends primarily on the depth that must be reached by light. Because of the shape of the absorption spectrum of the main chromophores in tissue, haemoglobin and melanin, the penetration depth increases with the wavelength in the visible and near-infrared spectral regions.[79]

Lasers present a very monochromatic range of wavelengths, which allows the selection of the optimum wavelength for a maximum absorption of photosensitizer, depending on its chemical structure. When the wavelength of a laser is selected in accordance with the absorption of the photosensitizer used, it is possible to generate a high production of ROS. It is important to note that a laser is not necessary for irradiation although it is convenient for endoscopic applications.

Light can be produced and controlled electronically in different ways. In light-emitting diodes (LEDs), light is produced by a solid-state process called electroluminescence. LEDs comprise a directional light source, with the maximum power emitted perpendicularly to the surface.[81] These apparatus are compact and lightweight, and require significantly less energy to produce the desired wavelength of light. The equipments have been manufactured to produce light of various wavelengths including 630, 670, and 690 nm, which could be used in PDT procedures for flat surface illumination. Light delivery for treatment of large surface areas, such as treatment of skin diseases, may also be effectively accomplished using fluorescent lamps. These could be of particular value in the activation of topically administered photosensitizers in sites where the limits of light penetration are not a key concern.[81] Wavelengths of 630, 670, and 690 nm represent interesting choices depending on the photosensitizer used in the respective PDT application, because these values correspond to the spectral window of significant light absorption of second-generation photosensitizer.

Associations of PDT with other Therapeutic Techniques

The effects of some monotherapies (PDT or chemotherapy, for example) have been compared with the results obtained by the combination between different therapies (PDT plus a specific chemotherapy drug, for instance) on cancer cells.[82–85] Indeed, selectively localized photosensitizers and appropriate doses of light combined with low doses of chemotherapeutic drugs represent a promising treatment strategy for cancer. Combinations of PDT and drugs would not only destroy cancer cells more efficiently but would also reduce the noxious side effects of chemotherapy, as a function of the lower quantity of chemotherapy required to obtain the desired effect. In this way, the additive and synergistic effects of combination treatment have been found in several trials.[82,83]

Combination of low-dose cisplatin with radiotherapy or PDT has been considered an important novel cancer treatment, as cisplatin is one of the most widely used anticancer drugs.[86,87] PDT is also considered complementary to radiotherapy by some authors, and would be better suited to treating larger tumours.[88] This kind of association is very plausible, because PDT presents a low complication rate and morbidity, achieving an increased median survival, as well as an improved quality of life even in patients with reduced performance status.[89]

PDT can also be applied in combination with other treatments (hyperthermia, ionizing radiation, electrotherapy, chemotherapeutic drugs) or with other agents. Indeed, it was shown that hyperthermia and PDT give synergistic killing effects. Another approach is to administer glucose in order to decrease the tumour pH, as the low pH value of tumours plays a role for the selective uptake of photosensitizers, such as Photofrin II. Likewise, interesting synergistic effects are obtained when PDT with Photofrin and an electric field are associated, even with a low number of exposure steps.[79]

In this context, it is interesting to mention the novel technology named photochemical internalization (PCI) for light-induced delivery of macromolecules of ALA.[79] The principle of PCI is to localize the photosensitizer, together with the drug or gene of choice, in endocytic vesicles within target cells. Irradiation of cells with light of a specific wavelength will excite

the photosensitizer to produce ROS, which subsequently disrupt the membranes, resulting in drug release.[90]

Porphyrins and Phthalocyanines: Macrocycle Compounds

The macrocycles are versatile compounds that can act as powerful ligands, as a result of the so-called 'template effect'. This effect provides great thermodynamic and kinetic stabilities to the metallic complexes.[91,92] Tetraazamacrocycles (Fig. 4), for example, stabilize various metallic cations in a significant way, because each one of the four nitrogen atoms represents an independent coordination site, producing very stable coordination compounds. It is relevant to register that even unusual oxidation states of metallic centres, such as iron(I) and iron(IV), are stabilized by the tetraazamacrocyclic ligands.[91,92] This stability, together with the versatility of possible applications and the evident biological relevance, has stimulated various studies about these compounds. Macrocycles have been employed in surface modification (thin films),[93,94] supramolecular systems (super molecules),[95,96] reconstitution of haemoproteins,[97–99] model complexes of haemoproteins, biosensors, and others.[100,101]

Porphyrins and Metalloporphyrins

The porphyrins and metalloporphyrins (Fig. 5) are very relevant tetraazamacrocyclic compounds widely encountered in nature. These compounds participate in important biological processes, such as photosynthesis. They represent active centres of haemoproteins, such as haemoglobins, myoglobins, and cytochromes, being essential in the respiration and electron transport chain. For example, iron protoporphyrin IX, the prosthetic group of haemoproteins, is involved in various biological processes: oxygen transfer, electron transfer, etc.

Recently, the importance of porphyrins and metalloporphyrins as therapeutic drugs has increased significantly. Many porphyrins, particularly derivatives of TMPyP (meso-tetrakis(*N*-methylpyridiniumyl)porphyrin), may exhibit activity against HIV.[102,103] Most studies have focussed on the high photostability and high affinity of porphyrins for tumour cells.[104] The effectiveness of porphyrins used as therapeutic drugs in PDT has been widely recognized.[105,106] As porphyrins and porphyrinoids are known to target tumour tissue, these compounds are investigated with great interest in the medical and pharmaceutical industries.[41] PDT through the accumulation of endogenous porphyrins, such as protoporphyrin IX (Fig. 3e), has been employed in skin with lesions after incubation with compounds like ALA.[107] The effort with respect to the synthesis, characterization, and reactivity of new macrocyclic compounds developed by several groups has generated very interesting works about the properties of the new porphyrins.[108–125] This effort includes trials in vivo about the excretion of porphyrins of interest in PDT[126] as well as studies on interactions of water-soluble porphyrins with nucleic acids.[127] Another interesting approach is the use of porphyrin molecules immobilized in oxidized nanoporous silicon matrix (pSi). This synthetic material is produced by electrochemical etching of monocrystalline silicon, which is able to provide an efficient electronic energy transfer to molecular oxygen. The consequent production of singlet oxygen in a gaseous and liquid environment propitiates the potential application of pSi in PDT.[128]

Studies have shown that porphyrins are incorporated into micelles, lipid bilayers, and other model systems of biomembranes. Actually, porphyrins can be solubilized and monodispersed in micelles. It seems that porphyrin macrocycles preferentially localize inside the hydrophobic core of micelles, even when these porphyrins present polar or charged peripheral substituents. Protoporphyrin IX (Fig. 3), for example, is described to reside in the hydrophobic region of a bilayer model membrane despite the two polar propionate chains. However, haematoporphyrin, which is substituted by two additional alcohol groups, adopts a different orientation and interacts with the polar micelle headgroups. In fact, instead of being embedded in the hydrophobic interior of a model membrane, porphyrins with charged substituents are also described to bind peripherally to surfactant surfaces of opposite charges.[41] Furthermore, in biological medium, metalloporphyrins can be coordinated by several biological molecules that can act as ligands, such as phosphate.[129] This is an interesting point because several metalloporphyrins, such as Sn(IV)TMPyP, do not aggregate, probably because of two axial ligands, and preserve their photosensitizing ability.[130] Therefore, the location of porphyrin photosensitizers in a biological membrane depends strongly on their peripheral substituent groups as well as their axial ligands, which determine solubility, chemical affinity, redox potential, and other properties of these macrocycles.

Porphyrin Aggregation and Molecular Interaction

Aggregate formation has been observed in various environments for chlorophylls, chlorins, and several synthetic porphyrins. The phenomenon of aggregation and dimerization of porphyrins and metalloporphyrins plays a significant role in their photophysical behaviour in aqueous solutions.[131–134] Porphyrins and their derivatives form distinct types of aggregates. These different kinds of aggregation can be induced by several conditions such as pH changes, ionic concentration modification, monomer functionalization, and surfactant addition.[135] The aggregation of some kinds of porphyrins used as drugs, such as benzoporphyrins, is associated with an inadequate biological delivery in clinical use.[136]

Self-assembly and self-organization that occurs with diverse compounds of biological interest, such as porphyrins, are natural and spontaneous processes, occurring mainly through non-covalent interactions such as van der Waals, hydrogen bonding, electrostatic interactions, and metal–ligand coordination networks.[137]

The photochemichal yield of singlet molecular oxygen ($^1O_2$) from porphyrins in different states of aggregation has been investigated in the literature.[79] These studies have shown that the photoexcitation of the clinically used Photofrin produces singlet molecular oxygen with significantly lower yields than photoexcitation of Hp. It was concluded that the fluorescence quantum yield and the singlet oxygen quantum yield of an aggregated sensitizer are remarkably lower than those obtained from the monomeric form.[79]

In this context, it is interesting to note that the binding of photosensitizers to biological molecules must be considered, because the confinement in a biological molecular assembly, which necessarily occurs, alters the photophysical properties of the photosensitizer.[138,139] In fact, the complexation with target molecules in cells or with transport vehicles should be an important influence on the physicochemical properties of the photosensitizer, especially at low concentration of porphyrins,

where the aggregation process is at a minimum or inexistent. Changes in photophysical behaviour (changes in absorption, fluorescence, kinetics of deactivation of the excited states, and generation of singlet oxygen) of porphyrins, metalloporphyrins, and other porphyrinoid sensitizers induced by their interaction with biopolymers, such as proteins and nucleic acids, as well as with transport vehicles such as liposomes or synthetic sensitizer carriers (for example, cyclodextrins and calixarenes) have been described.[138,139]

Action spectra, adjusted for penetration spectra through tissue, show the optimal wavelength for killing cells at a given depth below the tissue surface. Below ~2 mm, blue light in the Soret band (~410 nm) is more efficient with porphyrin photosensitizers than red light. This fact is due to the high excitation coefficient of porphyrins in the blue region, which is ~20 times larger than that reached in the red region.[75]

The interaction with surfactants and the monomer–aggregate equilibrium of porphyrin derivatives in micellar systems has also been addressed on a regular basis, as surfactants and others counterions may induce dimerization and aggregation of free-base ionic porphyrins.[140–143] The formation of aggregates alters significantly the physicochemical properties, which can be observed through the changes in their UV-visible spectra, quantum yield, lifetimes of singlet and triplet states, and, consequently, in the production of singlet molecular oxygen.[144–145]

This surfactant–porphyrin interaction is especially interesting for some water-soluble ionic porphyrins, because the ionic charges in the porphyrin substituents make the process of self-aggregation of these macrocyclic monomers in the absence of surfactants difficult. For example, we can mention TMPyP, as this cationic porphyrin presents four cationic substituent groups. These groups produce a substantial electrostatic repulsion between the porphyrin units, precluding, as a consequence, TMPyP self-aggregation, which remains, therefore, as a monomer independently of pH conditions.[144–146]

However, studies focussing on anionic surfactants have also been developed. The description of the aggregates formed by the anionic tetra(4-sulfanatophenyl) porphyrin ($TPPS_4$) with ionic and non-ionic surfactants has identified the formation of H- and J-aggregates, which are characterized by face-to-face or side-by-side arrangement of the porphyrins, respectively.[143] The spectroscopic properties of this porphyrin are sensitively affected by the aggregation phenomenon. Actually, UV-visible electronic absorption spectra are distinctly modified by the different kinds of aggregation. In the case of J-aggregates, a red shift of the Soret band is expected, whereas the H-aggregate produces a Soret band blue shift. In fact, a Soret band centred at 490 nm is observed together with a Q-band around 710 nm for $TPPS_4$, in the presence of the cationic surfactant cetyl trimethyl ammonium chloride.[143]

Mishra and coworkers[135] have reported the pH-induced aggregation of chlorin p_6, a potential photosensitizer for PDT. Aggregation was observed in the pH range between 7.0 and 3.0, but the aggregates dissociated in more acidic solutions. This process was attributed to successive protonation of the three anionic carboxylate groups and two ring nitrogen atoms, similar to the treatment developed by Gandini and coworkers.[143] The increase of hydrophobicity at pH values slightly lower than 7 produced the aggregation in this pH range. It could be a causal factor of the selective localization of porphyrins and chlorins with ionic side chains in tumour cells with a somewhat lower extracellular medium pH.[135]

Moreover, it is important to register that for the development of clinically useful porphyrin drugs, it is essential to characterize porphyrin–biological membrane interactions and to determine the factors that modulate such a relation.[41] In this way, the widespread studies focussed on the interaction with surfactants can be considered models of the interaction of porphyrins with biological membranes.

Several works have established that the flexibility of a macrocycle, such as porphyrin, is decisive in determining the chemical properties and the reactivity of the respective metallic complexes.[89,147–158] Moreover, factors such as coordination number, type of axial ligands, spin state, spatial orientation of axial ligands, and dielectric constant of solvent can drastically alter the properties of metallic complexes in spite of their similar molecular structures.[89,147–158] For example, we can mention the different species of hemichrome (bis-histidine complex) found in ferric forms of the giant extracellular haemoglobin of *Glossoscolex paulistus* (HbGp), depending only on the reciprocal orientation of the same axial ligands.[159,160] Furthermore, the complexity of the autoxidation behaviour presented by this giant haemoglobin, depending on the medium conditions, denotes that the relationship between the spatial conformation of globins and the flexibility of the haem pocket is not trivial, constituting a challenging topic in the chemistry of haemoproteins.[161,162] In fact, several recent works have focussed on the structural properties of HbGp in order to contribute to the understanding of the physicochemical properties of this HbGp as well as its structure–activity relationship.[163–166]

Furthermore, the comprehension of the photochemistry of metalloporphyrins has long been important in biochemistry as a function of their central role in photosynthesis, biological redox processes, and oxygen transport. Despite this fact, little is known on the kinetically labile, water-soluble porphyrin complexes, especially from the viewpoint of their photoinduced properties. Depending on their size, charge, and spin multiplicity, metal ions can fit into the central role of the porphyrin ring, forming regular metalloporphyrins. In a different way, several of them are located out of the ligand plane. This latter kind of structure induces special photophysical and photochemical features that are characteristic of various porphyrin complexes.[167] Thus, only with an in-depth study of the physicochemical properties of these macrocyclic systems, will the mechanisms of action of the photosensitizers be clearly elucidated, favouring the advancement of their application in PDT.

It is interesting to mention the structural correlation between porphyrins and phthalocyanines. Phthalocyanines are tetraazaporphyrins, i.e. porphyrazines with four additional benzene groups. In other words, phthalocyanines are tetrabenzoporphyrins with the substitution of the four *meso*-carbons present in the α-γ and β-δ axis of the porphyrin macrocyle by four nitrogens. Therefore, phthalocyanines are tetrabenzotetraazaporphyrins or simply tetrabenzoporphyrazines (Fig. 4).

Phthalocyanines and Metallophthalocyanines

Phthalocyanines were first synthesized in 1907 during a study of the properties of 1,2-cyanobenzamide. Linstead synthesized a vast range of phthalocyanines in the 1930s, and the X-ray analysis was later conducted by Robertson. Metallophtalocyanine complexes, especially Cu phthalocyanine, are produced in industry on a large scale (~50000 t per year). These complexes have long been used as blue-green dyes and pigments. Recently, the applications of metallophthalocyanine

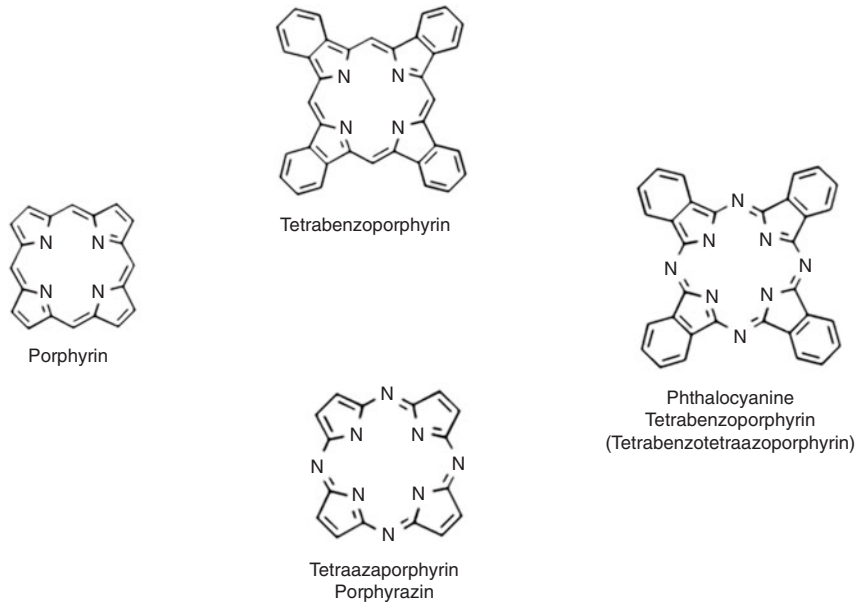

Fig. 4. Illustration of the structural relationship existing between porphyrins and phthalocyanines. Phthalocyanines are tetraazaporphyrins, i.e. porphyrazines with four additional benzene groups. In other words, the phthalocyanines are tetrabenzoporphyrins with the substitution of the four *meso*-carbons present in the α-γ and β-δ axis of the porphyrin macrocyle by four nitrogens. Therefore, phthalocyanines are tetrabenzotetraazaporphyrins or simply tetrabenzoporphyrazines.

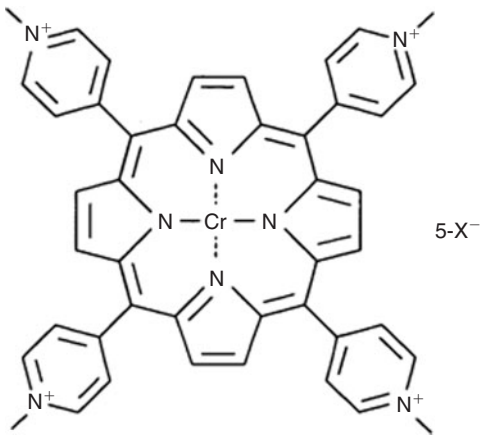

Fig. 5. Structural representation of a metalloporphyrin.

complexes have expanded to areas such as photosensitizers in PDT, photoconducting agents in photocopying machines and electrocatalysis.[168] The literature presents several interesting articles based on synthesis, characterization, and reactivity of phthalocyanines and metallophthalocyanines.[143–146,169–182] However, mainly in the last years, the studies about the application of these compounds in PDT have gained more emphasis. Aluminium phthalocyanine chloride is an example of a metallophthalocyanine with good preliminary results regarding applications in PDT.[168] Some new phthalocyanines, for instance, have been applied as photodynamic agents for the inactivation of microbial pathogens, such as *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*.[183]

Phthalocyanines are one of the major types of tetrapyrrole derivatives showing a wide range of applications in materials science, medicine, and catalysis. Over the past two decades, phthalocyanines have been extensively studied as an important class of third-order non-linear optical materials because of their extensively delocalized two-dimensional 18 π-electron system, their structural flexibility, their exceptionally high thermal and chemical structure, and their potential for use in photonic applications.[184,185] Indeed, phthalocyanines comprise a class that has been used in many areas of photonics, such as optical memory, optical power limiting, optical switching, and photomedicine.[184,185]

Phthalocyanines, together with chlorins, are considered second-generation compounds regarding their application as photosensitizers in PDT.[186] Indeed, phthalocyanines are an important group of porphyrin-like compounds, known for their exceptional stability and light absorption properties in the red or near IR region (molar absorption higher than 10^5 M^{-1} cm^{-1} for the longest wavelength absorption band often located as 650–700 nm).[187] They undergo successive electron oxidations and reductions, suggesting possible applications as photoredox sensitizers. The main limitation of phthalocyanines as photosensitizers is their low solubility even in organic solvents. Dissolution in water can be achieved by introduction of charged groups in the periphery of this macrocycle, as has been clearly shown with sulfonatophthalocyanine.[187] A second alternative to increase phthalocyanine hydrophilicity, associated only with metallic complexes, consists in the addition of an axial ligand with high polarity to coordinate to the metallic centre of this macrocycle.[42]

In any case, it is important to note that some phthalocyanines, such as zinc phthalocyanine, are at least two times more efficient than well-known sensitizers such as methylene blue and possess a very low cytotoxicity and high capacity to penetrate the cellular membrane.[188]

Phthalocyanines can be easily synthesized with a variety of different side groups that alter their molecular charge and solubility, modifying the uptake by tumour cells. The sulfonation of the side chain increases their solubility in water, implying that the uptake kinetics and cell retention are quite different for hydrophilic and hydrophobic phthalocyanines. This distinct

behaviour for each phthalocyanine regarding its polarity will directly affect the mechanism of cell death provoked by PDT, even without altering the light source. Photosensitizers with highly negative charges, for instance, are internalized by an endocytic mechanism due to the repulsive forces between these compounds and the plasma membrane, which presents a great number of negatively charged groups.[189]

The intense effort of various research groups to synthesize distinct phthalocyanines in order to apply these photosensitizers in PDT is justified because these macrocyclic compounds exhibit a long lifetime in the triplet excited state, resulting in a highly efficient $O_2(^1\Delta_g)$ production.[42] Furthermore, metallophthalocyanines present very accessible synthesis procedures as a result of the versatility of the phthalocyanines as ligands. Indeed, water-soluble nickel, cobalt, copper, zinc, and aluminium phthalocyanines have been synthesized, presenting promising results in applications against cancer cells.[190]

Although it is generally considered that metalloporphyrins and metallophthalocyanines present similar physicochemical and spectroscopic properties, they have many important differences. These divergent points are exhibited by several coordination compounds, such as manganese complexes. One major difference between manganese porphyrins and manganese phthalocyanines is related to the different spin state of both metallic complexes. The manganese phthalocyanines present an intermediate spin state, four-coordinate planar complex, whereas the manganese porphyrin is a high-spin complex that is most likely non-planar.[191]

Phthalocyanine Aggregation

It is well known that phthalocyanines exhibit a high aggregation tendency and can form dimeric and oligomeric species in solution owing to their extended π system. It has been shown that this molecular association greatly influences the intrinsic nature of macrocycles, including their spectroscopic, photophysical, electrochemical, and non-linear optical properties. A substantial number of investigations have focussed on the aggregation behaviour of substituted phthalocyanines in various solvent systems in which the aggregation number and some thermodynamic parameters were determined.[178,179,185]

Sulfonated metallophthalocyanines (MPc) complexes, for instance, frequently form dimers or higher aggregates in solution.[192] Aggregation in these complexes is easily characterized by UV-visible spectroscopy. Phthalocyanines aggregate owing to electronic interactions between rings of two or more molecules. J-aggregates have been assigned to a red-shifted band near 750 nm, whereas a blue-shifted band around 630 nm is attributed to H aggregates. MPc photosensitizers that form dimers and aggregates show lower photosensitization efficiency, because aggregation reduces the lifetime of the MPc's excited state. This effect is most probably due to enhanced state dissipation without radiation, and therefore lower quantum yields of the excited states and of singlet oxygen generation. The degree of sulfonation, isomeric composition, and the nature of the central metal ion affect the extent of aggregation. Biological environments support monomerization of phthalocyanines.[192]

The degree of aggregation in water increases with lipophilicity and, consequently, the prevalence of the less sulfonated fractions in solution are expected to increase aggregation.[163] In this context, it is important to mention the interesting work of Kuznetsova and coworkers,[178] which has contributed to the understanding of the influence of the degree of sulfonation in sulfonated phthalocyanines in their aggregation and singlet oxygen quantum yield in aqueous solution. In fact, steric hindrance is as important as the electronic properties in determining the tendency towards aggregation of the phthalocyanines.

PDT is a very auspicious clinical technique with recent advancements in the mechanisms of subcellular localization of photosensitizers,[193,194] involving strategies for photosensitizer delivery.[195]

Conclusions

The present review emphasizes the relevance of the recent work regarding the properties of photosensitizers employed in PDT. Important macrocyclic compounds, such as porphyrins and phthalocyanines, with or without metallic centres, have been widely tested in order to obtain new photosensitizers with lower skin photosensitivity and other collateral effects. PDT can be used as a unique clinical procedure or associated with other techniques against cancer, such as chemotherapy and radiotherapy. These applications are especially important in more complex clinical cases, a fact that denotes the versatility of this therapy. The understanding of the mechanism of photodynamic action is an important prerequisite to the advancement of PDT and it requires a wide analysis of all steps of this procedure. In this way, it is relevant that all the professionals interested in this interdisciplinary area can understand the structure–function relationship of the most used photosensitizers, such as porphyrins and phthalocyanines, as well as the photochemical principles of their respective photodynamic action, as PDT clearly increases median survival and quality of life even in patients with low performance status.

Therefore, the versatility of PDT makes this technique a powerful medical tool in order to combat several types of tumour as well as other kinds of disease. It is important to reinforce that the future perspectives are really auspicious for this therapy, because the photodynamic treatment can be used in an isolated way or in association with traditional methodologies. This topic represents one of the principal advantages of PDT because, even in the cases where PDT cannot act alone, the association with chemotherapy and radiotherapy decreases significantly the necessity of high applications of these treatments, which, as is well known, cause physiological damage as well as decreasing the quality of life of various patients.

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