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Review

Nanotechnology, Light and Chemical Action: an Effective Combination to Kill Cancer Cells

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Photodynamic therapy (PDT) is a minimally invasive and effective procedure for treatment of cancer, based on the combination of a drug (photosensitizer, PS), light (visible or near-infrared, NIR) and induced local formation of reactive oxygen species (ROS) and radicals. Despite its less significant side effects as compared with conventional therapies, many efforts still are been focused on enhancing the selectivity and efficiency of PSs and thus, of commercial drugs. Nanotechnology is providing many interesting possibilities and tools to develop drug delivery systems (DDS) and multifunctional platforms for therapy, diagnosis and theranostics. More recently, their effectiveness against tumor cells and tissues is being improved by combining the synergic effects of chemotherapeutic agents and other therapies, making them more interesting therapeutic alternatives. Accordingly, this review is focused on the recent contributions of nanotechnology on PDT, converging to the development of DDSs and multifunctional systems and their application for cancer therapy.

Keywords: photodynamic therapy, photosensitizer, drug delivery systems, nanocarriers, cancer

1. Introduction

Cancer is one of the most prevailing and fearful diseases in modern society, responsible for about 7.6 million deaths in 2008, and prevision to escalate to 13.1 million in 2030.[1] In fact, it refers to a group of diseases characterized by abnormal and uncontrolled growth of cells that invade a particular tissue or organ in an aggressive manner, and is capable to spread to other parts of the body, thus characterizing the so called metastasis process. According to the specialized literature, there are more than 100 different types of cancer and about 13 million of new cases are diagnosed every year.[2]

Generally is treated by chemotherapy, radiotherapy and surgery, techniques considered invasive and potentially capable of promoting serious short and/or long-term side effects. For example, those therapies can cause nausea and vomiting, disorders in the immune system, severe damage to epithelial surface, infertility, swelling of soft tissues, mutilation and other undesirable side effects.[3] Thus, efforts are being focused in their improvement and refinement, especially to minimize side effects. In fact, many non-conventional, less invasive, more efficient and cost-effective treatments are being developed.

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Photodynamic therapy (PDT) is an example of an alternative method for treatment of cancer that minimizes most of the unwanted side effects of conventional therapies. PDT is based on the combination of three factors when promoted by the so-called mechanism type II: (*i*) a photosensitizer (PS) agent, a drug that can be activated by light; (*ii*) irradiation of the affected region with light of appropriate wavelength, typically in the visible or near-infrared (NIR); and (*iii*) presence of oxygen. During irradiation, highly reactive species, such as reactive oxygen species (ROS), capable of causing direct damage to biomolecules, triggering the death of tumor cells, are generated *in situ*.[4] Also, the excited photosensitizer itself can be reactive enough to produce radical species or promote the direct oxidation of biomolecules, then leading to cell death by the type I mechanism. In short, the combination of nontoxic components (PS + oxygen) with low energy light generates cytotoxic species making possible the treatment just of the irradiated volume, in contrast with chemotherapy drugs that induce systemic toxicity, and ionizing electromagnetic radiation therapy that damages neighboring normal tissues.

The success of PDT is been possible thanks to a multidisciplinary effort involving researchers from several areas. Physicists have been working on the development

of new radiation sources (laser and light-emitting diode, LED). Chemists are contributing to the development of new molecules and formulations with suitable properties and to the understanding of the interaction of light with PS and biological systems, as well as the photophysical processes induced by irradiation. Biologists, biochemists and pharmacists are focusing their attention on the understanding of the mechanisms of transport, interaction and action of PSs in biological systems, in parallel with the possible toxic effects that they may induce. Finally, medical researchers are being responsible for the application and evaluation of the clinical efficacy of new PSs and sources of radiation in advanced tests, commonly using animal models and eventually patients, leading to the development of optimized clinical protocols. Although each of those areas seems to be performing restricted roles, a comprehensive knowledge and a multidisciplinary approach are needed to ensure the advancement of research on PDT. Similar are the cases of many other areas, but is particularly true in the case of most recently created areas, such as nanomedicine and biomedical engineering.

In the particular case of chemistry, understand the photophysics and photochemistry of PS, as well as its mechanism of action and interaction in biological systems, are fundamental for the design of more efficient and selective photosensitizers, exhibiting enhanced pharmacokinetic and targeting properties. However, hardly the actual behavior and biological activity of a molecule can be anticipated thoroughly. Accordingly, a variety of molecules with appropriate structural and photophysical properties have been synthesized and tested for PDT application.

More recently, nanobiomaterials increased the possibilities to develop new pharmaceutical formulations, enhancing the efficiency and bioavailability while reducing the toxicity and side effects of well-established and newly developed drugs. In the particular case of PDT, the combination of PSs and nanosystems has shown to be very promising. The major achievements leading to the development of liposomes, micelles and polymeric nanoparticles for PDT application in the last five years, as well as of nanosystems synergically combining PSs and chemotherapeutic or PDT agents with other therapies, in order to overcome the eventual drawbacks and extend the frontiers are the main focus of this review.

2. Photodynamic Therapy

Photodynamic therapy has been used for cancer therapy for more than 25 years, and its successful application for treatment of several types of cancer has been recently reviewed.[5-8] Nowadays, PDT is also been employed for treatment of diseases, such as leishmaniasis,[9] psoriasis,[10] age-related macular degeneration,[11] as well as in cardiology,[12,13] urology,[14] immunology,[15] ophthalmology,[11,16] dentistry,[17-19] and dermatology.[9,20-22] The combination of light and PS has been promising for treatment of bacterial, fungal, parasitic and viral infections too.[23-27]

Broadly, the clinical procedure can be described by the scheme shown in Figure 1a. The PS is administered intravenously, orally or topically, and accumulates in the tumor tissues, which is then irradiated with a light source of appropriate wavelength (typically visible and NIR light). Generally, a short-lived excited PS in the singlet state is generated upon absorption of a photon that converts to a more long-lived triplet state (PS*) after intersystem crossing. Then, the excited PS* may transfer a proton or an electron to other molecules and biomolecules to form a radical cation or anion, respectively, which then reacts with oxygen to produce reactive oxygenated species, such as superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide (type I mechanism). Alternatively, in the type II mechanism, the excess of energy of PS* is transferred to ground state oxygen molecules, generating reactive singlet oxygen ($^1O_2$).[1] The amount of ROS generated can be controlled by the light dose in order to promote enough cell and tissue damage to induce necrosis and apoptosis, indirectly stimulating the production of inflammatory mediators. In addition, PS* itself may also be toxic to the target cells through the so-called type III reaction, in which the PS* reacts directly with biomolecules (nucleotides in DNA and protein residues) through an oxygen-independent pathway.[28] The three mechanisms are illustrated in the scheme shown in Figure 1b.

PDT can also be combined with other therapies, such as chemotherapy, radiotherapy, hyperthermia and electrotherapy and has presented promising results in the treatment of several types of cancer, significantly reducing the side effects as compared to conventional therapies.[29] Furthermore, PDT can help releasing chemotherapy agents from acidic compartments, thereby increasing its availability at the respective intracellular sites of action, improving their efficacy. Khdair *et al.*[30] investigated the anticancer activity of doxorubicin (Dox) in combination with photodynamic therapy based on methylene blue (MB) in drug-resistant NCI/ADR-RES cells and a drug-resistant mouse tumor model.[31] Aerosol-OT™ (AOT, Fisher Scientific) and a naturally occurring polysaccharide polymer, sodium alginate, were used for synchronized delivery of the two drugs. This combination resulted in a significant inhibition of tumor cell proliferation, as a consequence of enhanced cytotoxicity and elevated local ROS production as compared to single

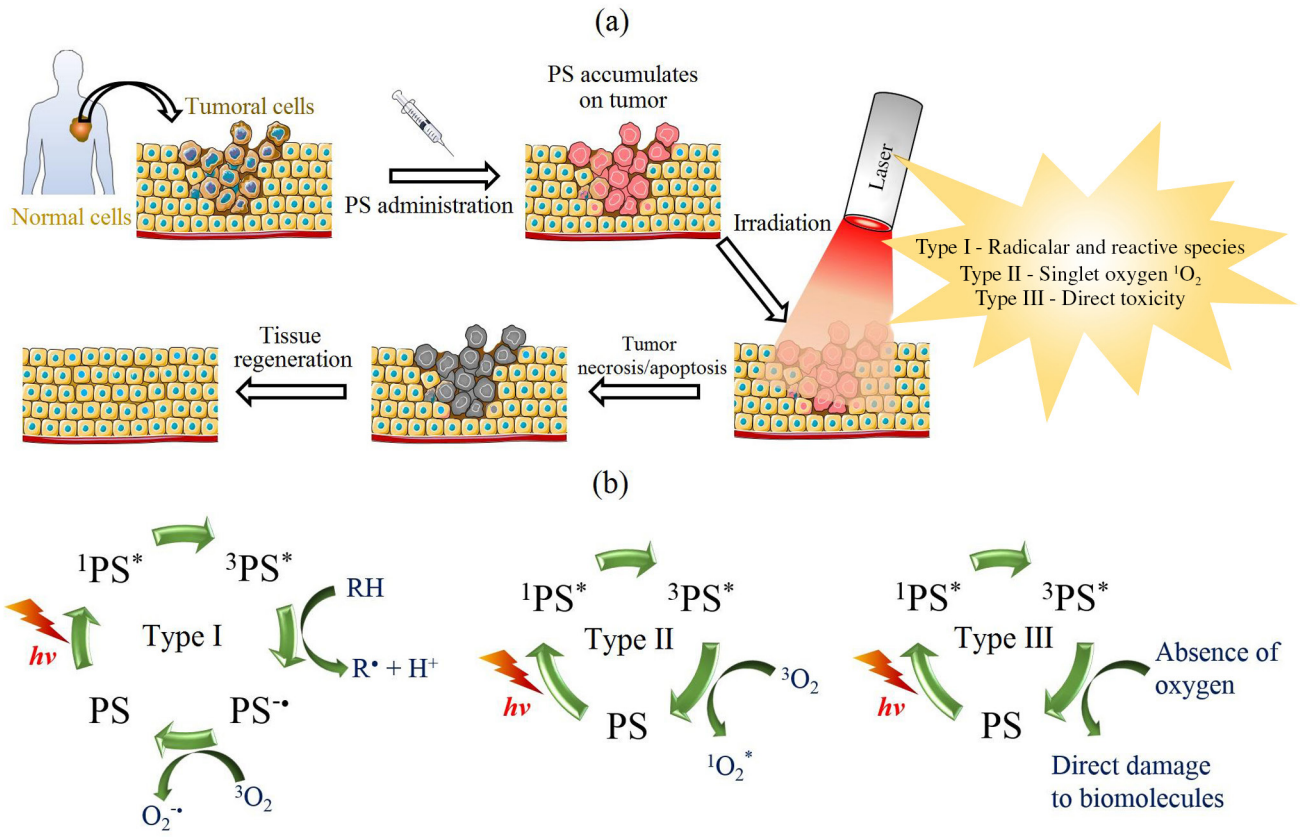


Figure 1. (a) Scheme showing a typical clinical procedure for application of photodynamic therapy and (b) the three possible mechanisms of action involving excited photosensitizer molecules.

drug treatment, suggesting its potential for treatment of drug-resistant tumors.

Although this elegant combination of drug and light seems simple, it depends on several factors including the light source employed, as well as the complex interaction processes in the body and the effective incorporation of drugs by tumor cells, the presence of oxygen, and the generation of singlet oxygen and/or other reactive species in the desired location and in high enough concentrations to promote the destruction of tumor tissues.[32] In fact, the success of PDT is mainly due to the development of new light sources with appropriate characteristics for the therapy,[33] allied to a better understanding of PSs interaction mechanisms with tumor cells, which have enabled the synthesis of new photosensitizers with higher photodynamic efficiency.

3. Photosensitizers

Photosensitizers are light-absorbing species which exhibit suitable energy and/or electron transfer properties. The main PSs currently in use for PDT are porphyrins and their analogs, such as chlorins, bacteriochlorins, and phthalocyanines, i.e., cyclic tetrapyrrolic aromatic structures like those shown in Figure 2. Their advantages and drawbacks have been recently reviewed in detail by Ormond and Freeman.[34] Examples of potential non-porphyrinic photosensitizers include Rose bengal (RB),

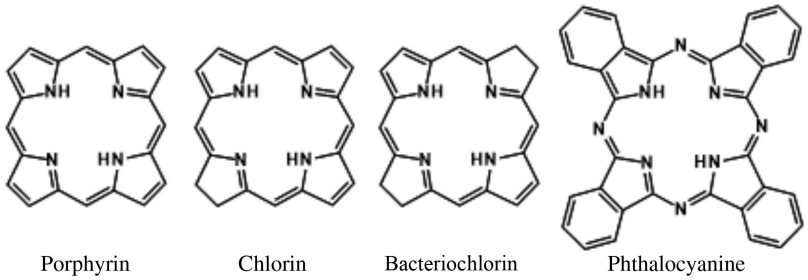
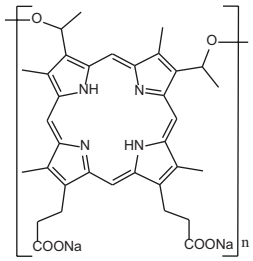
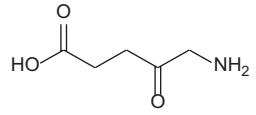
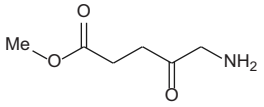
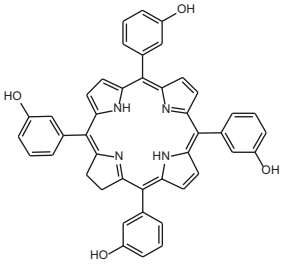
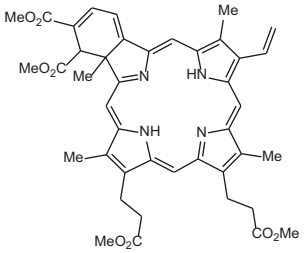
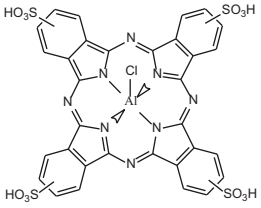
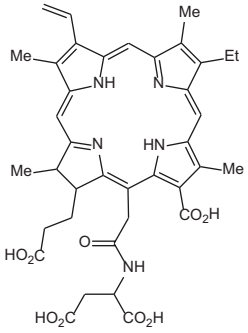
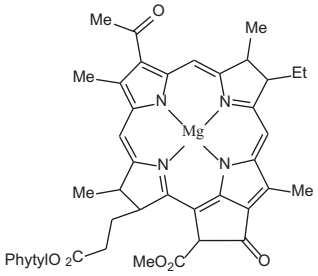
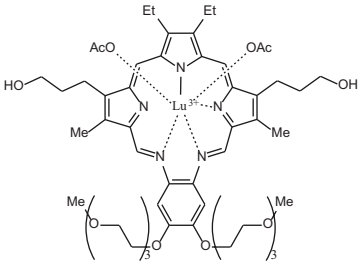


Figure 2. Scheme showing the basic structures of some porphyrinoid photosensitizers.

Table 1. List showing the trademark, molecular structure, nomenclature and main applications of most relevant photosensitizers approved for photodynamic therapy (PDT) treatment or in clinical trial

Trademark	Compound	Main application
Photofrin®	hematoporphyrin derivative (HpD) 	early- and late-stage lung cancers, esophageal cancer, bladder cancer, malignant and nonmalignant skin diseases, early-stage cervical cancer
Levulan®	5-aminolevulinic acid (ALA) 	actinic keratosis
Metvix®	methyl-5-amino-4-oxopentanoate (methyl aminolevulinate, MAL) 	actinic keratosis, basal cell carcinoma
Foscan®	*meta*-tetra(hydroxyphenyl)chlorin (*m*-THPC) 	head cancer, neck cancer
Visudyne®	benzoporphyrin derivative monoacid ring A (BPDMA) 	age-related macular degeneration
Photosens® (approved in Russia)	aluminum phthalocyanine tetrasulfonate (AlPcS4) 	stomach cancer, skin cancer, lips cancer, oral cavity cancer, tongue cancer, breast cancer
Laserphyrin® (approved in Japan)	*N*-aspartyl chlorin e6 (NPe6) 	early lung cancer
Tookad® (clinical trial)	bacteriochlorophyll a 	prostate cancer
Lutrin® (clinical trial)	motexafin lutetium 	prostate cancer, breast cancer, malignant melanoma

MB, acrydine dyes[35] and 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) derivatives.[36] Table 1 presents the PSs that have been approved or achieved clinical trial stage for treatment of specific types of cancer and other diseases. Their main characteristics, applications and limitations were described in detail in several recent reviews.[3,8,11,34,36-39]

Porfimer sodium (Photofrin®, Pinnacle Biologics) is one of the earliest clinically approved PDT agent and has received worldwide regulatory approval mainly for treatment of esophagus, lung and bladder cancer. It is a mixture of oligomeric porphyrin units (up to eight) linked by ester and ether groups activated by red light (630 nm).

Photons of this wavelength are able to penetrate just few millimeters in biological tissues. As a consequence, porfimer sodium is only suitable for superficial tumors or those that can be reached by endoscopic/fiber optic procedures.[36]

The 5-aminolevulinic acid (ALA)-based photosensitizers, like Levulan® (DUSA Pharmaceuticals, Inc.) and Metvix® (MAL; Photocure), are based on non-photoactive molecules by themselves (prodrugs), but show a preferential intracellular accumulation inside cancer cells where the active species is generated. In fact, they are metabolized in the heme biosynthesis cycle to porphyrin derivatives, such as protoporphyrin IX (PPIX) able to produce reactive species and large enough oxidative stress after irradiation to induce cell death.[28] Metvix® is based on a methyl ester precursor of Levulan® and presents greater penetration through the skin stratum corneum. Once inside the target cells, Metvix® is demethylated to ALA by the action of intracelular estearases.[39] Rollakanti *et al.*[40] have identified a number of cellular differentiation-promoting agents that increase the ability of epithelial cells to synthesize PPIX from exogenous ALA. Among them, the administration of low nontoxic doses of vitamin D lead to augmented tumor response to ALA-PDT, offering new perspectives for improved remission of cutaneous breast cancer metastases.

Among the PSs in clinical trials, Tookad® (palladium-bacteriopheophorbide; Steba Biotech) has presented promising results for prostate cancer treatment. This drug accumulates in the tumor vasculature inducing vessel occlusion and stasis upon irradiation, leading to tissue ischemia and necrosis and, eventually, tumor ablation. It presents a high molar extinction coefficient at maximum absorption wavelength of 763 nm ($\varepsilon_0 = 10.86 \times 10^4$ mol L^{-1} cm^{-1} in chloroform) where the light penetration depth is 4 mm as compared to 1.6 mm at 630 nm used for Photofrin®. Also, showed faster clearance from circulation of mice (15 min), preventing accumulation in tissues and photosensitivity, and a plasma half-life of about 20 min in mice.[39] Other photosensitizers in clinical trial or preclinical testing include Purlytin® (Trademarkia), Lutrin® (Pharmacyclics), Hypericin® (Aktin Chemicals, Inc.), chalcogenopyrylium dyes, phenothiazinium dyes (methylene blue, toluidine blue, nile blue) and derivatives, cyanines, etc.[34]

In recent decades, new compounds, particularly porphyrin derivatives with more appropriate photophysical and structural properties, have been synthesized in order to minimize/eliminate the side effects caused by conventional drugs, as well as increase the efficiency, selectivity and biocompatibility.[41-43] Typically, an ideal photosensitizer should present the following properties:[3,37,38] (*i*) PS synthetic route should be efficient and reliable to allow for excellent and reproducible batch to batch synthetic yields; (*ii*) the drug should be stable and the reconstitution process should be simple to perform when needed; (*iii*) the administration of the drug should be rather by topical than systemic way, either intravenous, intranasal or by oral ingestion, without toxicity or pain; (*iv*) ideally the PS should preferentially accumulate in the tumor tissue to maximize selectivity of therapy; (*v*) rapid accumulation and clearance of the PS is preferred allowing the treatment in a day associated with low systemic toxicity; (*vi*) the PS should be cytotoxic only in the presence of light; (*vii*) PS must have high quantum yield for ROS generation, mainly singlet oxygen; and, (*viii*) PS should have high extinction coefficients in the electromagnetic spectrum region, where the light presents maximum penetration in the tissues and enough energy to produce singlet oxygen (typically in the range of 600 to 900 nm).

Photosensitizers can be classified according to their synthetic purity, targeting/selective accumulation properties, chemical structure and generation. Porphyrin based PSs constituted the first generation and included hematoporphyrin and its derivatives named hematoporphyrin derivatives (HpD). Second generation PSs were developed aiming to solve deficiencies and limitations presented by the first generation drugs, and are constituted by various compounds including porphyrins, expanded porphyrins, and chlorophyll derivatives, and dyes with high absorption properties in the therapeutic window and low toxic side effects. Third generation drugs contain first and second generation PSs conjugated to biomolecules, like proteins, peptides and also nanocarriers, in order to improve selectivity and bioavailability.[37]

Substantial progress has been achieved in recent years considering both, the development of new potential PSs and radiation sources, leading to improved therapeutic efficiencies and clinical protocols. Significant success has been achieved in the synthesis of stable PSs absorbing light in the red and NIR region of the spectrum (650-800 nm), which penetrates deeper in tissues.[44] Some studies suggest that NIR light (typically between 700 and 1000 nm) presents lower photo-toxicity to normal cells and tissues, also affording penetration depths an order of magnitude larger than that of visible light.[45]

However, a key obstacle to be overcome is the low water solubility, low bioavailability and the tendency of several currently investigated PSs to form non-photoactive aggregates (dimers, trimers and oligomers). The formation of aggregates and precipitates not only reduces the photoactivity but can cause serious side-effects due to

clogging of arteries and veins.[46] Another challenge is to increase the selectivity of therapy. Although some photosensitizers accumulate in tumor tissues with certain selectivity, they also accumulate in healthy tissues resulting in undesired side effects, such as prolonged photosensitivity and photoalergic reactions. Accordingly, the development of new strategies to increase PSs solubility and selectivity is one of the main goals of current research in PDT.

One strategy to reduce PSs aggregation is the introduction of bulky and/or electrically charged groups in the periphery of the porphyrin ring in order to prevent π-stacking interactions by means of steric and electrostatic effects.[47] Santos *et al*.[48] described the synthesis and characterization of some functionalized chlorin derivatives hybridized with different substituted maleimides. Studies performed by nuclear magnetic resonance (NMR), ultraviolet-visible (UV-Vis) spectroscopy and also high resolution mass spectrometry (electrospray ionization time-of-flight, ESI-TOF; and matrix-assisted laser desorption/ionization-time-of-flight, MALDI-TOF) indicated that these compounds are immune to aggregation in solution. Photophysical and photochemical evaluations were also performed, showing that the new photosensitizers present singlet oxygen quantum yields in the 0.35 and 0.41 range, considered suitable for PDT application.

Studies to investigate the photophysical properties and the structure/activity relationship of compounds with potential application in PDT have been carried out[42,49,50] revealing fundamental aspects to develop strategies for further improvement of PSs. These studies showed that features, such as the amphiphilic character and the electric charge, influence significantly their interaction with the cell membrane, the cytolocalization and the singlet oxygen quantum yield, the main factors responsible for the photodynamic efficiency. All these factors are discussed in a review recently published by Benov.[7]

Amphiphilic photosensitizers with appropriate structural properties and characteristics to improve selectivity and cell interaction have been synthesized in the last five years,[51] and promising results have been obtained in *in vitro* and *in vivo* studies.[52,53] Another alternative that has presented interesting results is the conjugation of biomolecules, such as peptides, anti-bodies and folic acid, to the porphyrin ring in order to produce molecules with targeting properties. Han *et al*.[54] synthesized a PS conjugating PPIX to an amphipathic biodrug $(KLAKLAK)_2$ peptide through a short polyethylene glycol (PEG) linker, able to target mitochondria. The *in situ* generation of ROS led to significant decrease in mitochondrial membrane potential and cell death. Relative high accumulation in tumors, minimal systemic cytotoxicity and efficient long-term tumor inhibition *in vivo* were confirmed using a murine model. Antibodies or antibody fragments can also be conjugated to PSs in order to improve selectivity and biocompatibility.[55] Another strategy is based on the conjugation of polymeric chains to porphyrin derivatives. Nawalany *et al.*[56] prepared a 5,10,15,20-tetrakis(4-hydroxyphenyl)porphyrin (*p*-THPP) functionalized with PEG (350, 2000 or 5000 Da). *In vitro* studies showed that the presence of PEG side chains reduces the cytotoxicity of the porphyrin in the dark. Also, a phototoxicity dependent on the length of PEG chain was observed for the PEGylated porphyrins.

In recent years, the use of nanotechnologic strategies has improved the efficiency of PDT treatments by increasing the pharmaceutical efficacy of several drugs. Nanocarriers with suitable physico-chemical characteristics to protect the drug and improve selectivity and solubility has shown very interesting results for treatment of cancer and several others diseases. *In vitro* and *in vivo* studies confirmed an increase in efficacy of chemotherapeutic agents when incorporated in nanosystems, such as liposomes, micelles and polymeric particles.[57-60] The achievements employing this strategy on PDT research are the main focus of this review.

4. Photosensitizers Nanoformulations

Nanotechnology has given major contributions in recent years in the pharmaceutical area for the development of formulations to enhance solubility, bioavailability, uptake, biodistribution, metabolism and excretion of drugs, with consequent decrease in toxicity.[61] Among them, biocompatible and/or biodegradable nanosystems for transport and controlled release of drugs, such as lipidic, micellar and polymeric carriers, are the most widely explored alternatives.[4,62,63]

Drug delivery systems (DDS) offer several advantages over conventional formulations: they (*i*) protect the drug from premature degradation, (*ii*) increase drug solubility and circulation time in the bloodstream, (*iii*) improve intracellular penetration, (*iv*) improve drug delivery to selected cells and tissues, (*v*) prevent the drug efflux by multi-drug resistance pumps, and (*vi*) control the release of drugs from few days to few weeks.[64,65] Thus, the use of nanocarriers enabled the reduction of the number of administrations, which means greater comfort and better adherence to treatment, as well as consequent cost reduction.[4,61] Alternative administration routes, such as oral and transdermal, are feasible by using DDS. In addition, organs that cannot be treated by conventional drugs because they fail to cross biological barriers, such as blood-brain barrier, can be successfully reached.[66]

In recent years it has been reported that PSs can be combined with different micro and nanometric systems enabling the development of new photosensitizing agents (nanophotosensitizers) for PDT application.[6] Covalent conjugation or physical inclusion of photosensitizers to nanoparticle carrier systems changes the PS interaction with the biological medium, including cells, and consequently the photodynamic efficacy. Also, this strategy can facilitate the dispersion of lipophilic molecules, otherwise insoluble in aqueous media, making them compatible with the biological environment. The results are formulations with reduced photosensitivity to the skin and eyes, enhanced antitumor efficacy and increased passive tumor accumulation *via* enhanced permeability and retention (EPR) effect by tumor tissues.[67]

Nanosystems can also be employed to increase PSs selectivity for tumor cells by an active or passive mechanism. Passive targeting is achieved when the drug accumulates in tumor tissues due to nanocarriers specific characteristics, such as composition, size and surface properties, in addition to pathophysiological factors, such as the tumor microenvironment and EPR effect. In the active mechanism, the drug is delivered to specific target sites by molecular recognition process as a result of specific interactions of molecules/biomolecules present in nanosystems surface with biomolecules expressed in tumor cells.[68]

Several nanoformulations have been developed and evaluated by researchers around the world and some of them are already commercially available, such as the lipossomal formulations temoporfin (Foscan®, European Medicines Agency) and verteporfin (Visudyne®, Bausch & Lomb Inc.). Other promising systems include polymeric nanospheres and nanocapsules, micelles, carbon nano-platforms and even silica and metal particles, with or without active targeting molecules, such as proteins, peptides and aptamers.

4.1. Liposomes and micelles

Liposomes and micelles were the first nanosystems investigated to carry and delivery PSs in PDT. They have similar structures, characterized by a core/shell like system able to incorporate hydrophilic, hydrophobic and amphiphilic drugs, as illustrated in Figure 3.

Liposomes have been investigated since 1970 as carrier systems to improve the delivery of drugs to specific sites in the body, being the first class of nanoparticles used in medicine. A liposome is constituted by a lipid bilayer and can incorporate hydrophilic drugs in the aqueous core and/or hydrophobic drugs in the bilayer. Recognition groups also can be anchored to the bilayer surface to enhance the selectivity.[69] Liposomes and several other vesicular systems, such as niosomes, transfersomes and pharmacosomes, have been investigated and applied in immunology and dermatology, as vaccine adjuvant and brain targeting, as well as for treatment of eye disorders, infective diseases and tumors.[57,70]

Several methods are available for preparation of liposomes controlling the size and number of shells, but ultrasound and extrusion processing are the most widely employed methods nowadays. The selection of a suitable preparation method is dependent on photosensitizers' properties, such as the balance of hydrophobicity and hydrophilicity, the desired final particle size and the intended application.[71,72] Liposomes are capable to fuse with cytoplasmic membrane delivering the load of active molecules to the cell.[58]

Although liposomes and micelles have a similar core/shell like organization, some differences can be easily noticed on their structure and chemical composition. Micelles are monolayer structures formed by amphiphilic surfactants or block copolymer molecules in liquid-colloid dispersions, in which the hydrophilic head are in contact with the aqueous phase (typically the surrounding solvent) and the hydrophobic tails are directed to the center (Figure 3). They are generally used to carry hydrophobic PSs, which can be physically incorporated in or covalently bound to the hydrophobic core, to be delivered to tumors via passive or active targeting strategies.[67] Hydrophilic and amphiphilic substances also can be adsorbed on the surface or dissolved in the micelles shell (Figure 3) in order to tune their properties.

Polymeric micelles have been extensively used in recent years due to their higher stability as compared to micellar systems based on conventional surfactants. Their higher stability can be assigned to the presence of multiple hydrophobic interaction sites in the polymeric chain.[61,73,74] Polymeric micelles are frequently prepared by association of copolymers dispersed in an aqueous medium, forming particles with diameters below 100 nm. Alkyl chains or lipids may also be conjugated to the polymer in order to enable the formation of a hydrophobic core to dissolve poorly water-soluble drugs. In analogy to some detergents, the dispersion of polyethylene glycol-phosphatidylethanolamine conjugates (PEG-PEs) films in aqueous solution was shown to spontaneously generate 7 to 35 nm diameter micelles.[73,75]

Another alternative that has been recently explored involves the conjugation of suitable polymers and co-polymers to the four porphyrin ring *meso*-positions in order to obtain photosensitizers with star-shaped

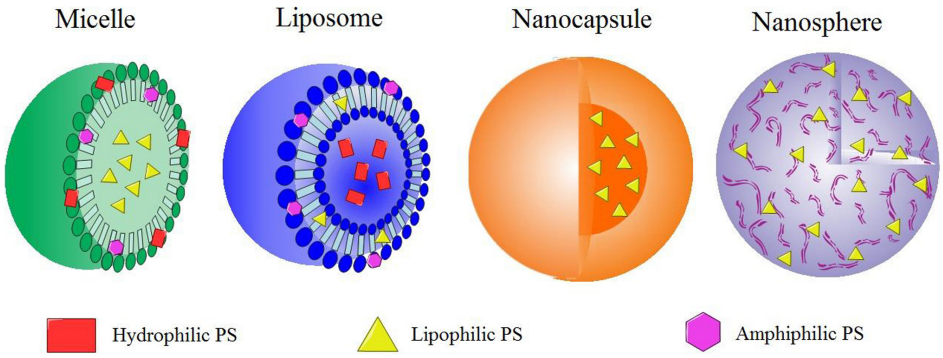


Figure 3. Schematic representation of some drug delivery systems investigated for photodynamic therapy (PDT) application, showing the preferential localization of hydrophilic, lipophilic and amphiphilic photosensitizers in them. Hydrophilic and amphiphilic photosensitizers (PSs) can also be incorporated upon adjustment on nanocapsules and nanospheres polymer composition (not shown).

structure prone to self-assemble, forming nano-micelles in water. The PS is the core and the polymeric chains interact more or less strongly, forming the shell at low critical micellar concentration (CMC). In general, these systems exhibit high singlet oxygen and high fluorescence quantum yields.[76-78] Interesting photodynamic efficiency against psoriasis[10] and tumor cells *in vitro*[79] has also been reported.

Liposomes and micelles have been extensively studied for PDT application showing that the chemical composition and size of these nanosystems and the presence of targeting ligands can modify some PSs fundamental properties, such as their aggregation state, interaction with biological systems, systemic circulation, pharmacokinetics and cytolocalization. All these factors can modulate PSs cytotoxicity (in the dark) and photodynamic efficacy. Thus, a greater PDT activity is observed when nanosystems with adequate characteristics are employed.

The main side effect reported in PDT is the prolonged photosensitivity, as described before. Encapsulation of PSs may also be promising to reduce or avoid its accumulation on skin and the photosensitivity, as demonstrated by Shieh *et al.*[80] They evaluated the cytotoxicity and antitumor effects *in vitro* and *in vivo* of poly(2-ethyl-2-oxazoline)-b-poly(D,L-lactide) diblock copolymer micelles loaded with *m*-tetra(hydroxyphenyl)chlorin (*m*-THPC) as photosensitizer, which showed no significant adverse effects *in vivo* in mice model. Interestingly, the micellar formulation presented lower skin phototoxicity after an extended delivery time, despite the similar antitumor effects as compared with free *m*-THPC.

4.1.1. Effects of liposomes and micelles on PSs aggregation, stability and photodynamic activity

The incorporation of photosensitizers in liposomes and micelles tend to decrease the aggregation and improve their solubility, stability and tumor-selective accumulation. In addition, the photophysical and photochemical properties of PSs can be changed by incorporation in micelles and liposomes, consequently, changing their photodynamic activity.[4] Spectroscopic studies realized by Chai *et al.*[81] showed that hydrophobic metallo-tetraphenylporphyrins (TPP; MgTPP and ZnTPP) incorporated in polyethylene glycol-block-poly(4-vinylpyridine) (PEG-*b*-P4VP) micelles possess higher photostability and better electron transfer capability than the free species. The approved PS, Photofrin®, was recently incorporated in micelles prepared with an amphiphilic chitosan derivative[82] and its photophysical properties and PDT activity evaluated. The encapsulated PS presented lower fluorescence quantum yield and fluorescence lifetime than free Photofrin® in solution, indicating that micelle is suppressing the photoactivity of the PS. However, the micellar formulation showed higher fluorescence and generated higher levels of ROS than free Photofrin® in *in vitro* experiments, inducing stronger phototoxicity and significant levels of apoptosis in human pancreatic cancer cells. These clearly indicate that the PS was effectively delivered to cells and concentrated in microenvironments, leading to less pronounced excited state suppression.

Garcia *et al.*[83] showed by absorption spectra, triplet excited state and singlet oxygen quantum yield measurements, that the incorporation of zinc phthalocyanine (ZnPc) and zinc hexadecafluorophthalocyanine ($ZnF_{16}Pc$) into liposomal formulations of dimyristoyl-phosphatidyl choline (DMPC), dipalmitoyl-phosphatidyl choline (DPPC) and distearoyl-phosphatidyl choline (DSPC) decreases the degree of aggregation of those photosensitizers. In addition, *in vitro* studies using human cervical carcinoma (HeLa) cells indicated that the photodynamic activity is influenced by the presence of aggregates and the composition of liposome bilayer.

Temoporfin (Foscan®, *m*-THPC) is an example of PS formulated using a commercially available unilamellar

liposomal delivery system based on dipalmitoyl-phosphatidyl choline/dipalmitoyl-phosphatidyl glycerol (DPPC/DPPG). The chlorin-based photosensitizer (Table 1) presents high hydrophobic character that favors its precipitation in biological media when not administered as a liposomal formulation. The liposomal formulation Foslip® (Biolitec AG) promotes reduced damage to healthy tissues and have higher efficacy and lower toxicity in the absence of light as compared to Foscan® and is in preclinical tests.[4] Its biodistribution, pharmacokinetics and photodynamic efficiency were evaluated by Lassalle *et al.*[84] The best antitumor response was observed 6 h after drug administration, when *m*-THPC was detected in both endothelial and parenchyma cells. According to Reshetov *et al.*,[85] Fospeg® (Biolitec AG), a *m*-THPC liposomal formulation based on polyethylene glycosylated liposome, exhibits higher photodynamic efficacy in tumor-grafted mice as compared with Foslip® due to the enhanced EPR-based accumulation in tumor cells associated with improved stability in the blood circulation and PS release properties. Another example is verteporfin (Visudyne®), a liposomal formulation approved for treatment of age-related macular degeneration (AMD), a disease caused by abnormal blood vessel growth on retina. Non-encapsulated verteporfin aggregates in aqueous solution, impairing the photodynamic activity and causing undesirable side effects.[36]

Photosensitizers other than *m*-THPC and verteporfin have also been investigated as liposomal formulations for PDT application. In a recent work published by Li *et al.*,[86] hypocrellin B (HB), a photosensitizer isolated from *Hypocrella bambuase* fungus sacs, was incorporated in multilamellar vesicles made of egg lecithin and cholesterol, showing promising results for PDT treatment of AMD. HB has low toxicity in the dark, a high metabolic rate *in vivo* and generates high concentrations of ROS. The stability, *in vitro* PDT efficacy and *in vivo* pharmacokinetics, and skin phototoxicity of the liposomal formulation were evaluated. The formulation presented high stability and *in vitro* photodynamic efficiency even after one year storage, associated with a short half-life (about 2 h) and nearly complete clearance and no phototoxicity 24 h after injection in mice. The encapsulation of HB in phosphatidylcholine liposomes also enhanced its phototoxicity *in vitro* against HeLa cells, probably due to a more efficient delivery and, consequently, higher intracellular concentrations, as reported by Zhou *et al.*[87]

Rocha *et al.*[88] described the PDT efficacy of chloro-aluminum-phthalocyanine encapsulated in liposomes for the treatment of female dog breast cancer cells *in vitro*. The high cell necrosis rate was accompanied by morphological alterations, not observed in the dark, as confirmed by optical and electron microscopy. The use of liposomal chloro-aluminum-phthalocyanine was used for treatment of different oral cancer cell lines (oral squamous cell carcinoma, OSCC; and hmn salivary gland tumor cell, HSG), and in OSCC tumors induced in nude mice as reported by Longo *et al.*[89] *In vitro* and *in vivo* assays led to disruption of tumor blood vessels and cells death mainly by necrosis.

4.1.2. Particles composition, size and charge

Particles size is an important factor controlling pharmacokinetics and biodistribution of PSs, especially when administered systemically. Generally, small particles are more easily incorporated by endocytosis and can also accumulate in tumor tissues by EPR effects. On the other hand, larger particles are recognized by the reticuloendothelial system, activating the immune system.[71]

It has been demonstrated that liposomes charge can modulate the interaction and uptake by tumoral cells influencing the PDT activity. Cationic DMPC liposomes and cationic gemini surfactant were utilized to incorporate *m*-THPC, increasing its uptake by various human glioblastoma cell lines (A172, DBTRG, LN229, U118) *in vitro*, leading to their total destruction after irradiation.[90] Cationic vesicles loaded with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) also showed better penetration into skin, delivering a higher concentration of ALA as compared to neutral or anionic liposomes.[76]

Several authors described that the incorporation of PSs in micelles can significantly decrease the tendency to form aggregates. However, the micellar composition also has an important role to preserve the PS in the monomeric form. This was demonstrated by Romero *et al.*,[91] who incorporated a lipophilic tetra-menthyl substituted zinc phthalocyanine (ZnMintPc) in micelles prepared with 12 different surfactants, namely the ionic detergents sodium dodecylsulfate (SDS), cetyltrimethylammonium bromide (CTAB) and *N*-hexadecyl-*N*-*N*'-dimethyl-3-ammonio-1-propane-sulfonate (HPS), the non-ionic detergents Tween® 20 and Tween® 80 (Sigma-Aldrich), polyoxyethylene 9 lauryl ether ($C_{12}E_9$), Brij® 30, Brij® 35, Brij® 97 and Brij® 98 (Sigma-Aldrich), and the triblock copolymers Pluronic® F-68 and Pluronic® F-127 (Sigma-Aldrich). The ability of the PS to generate singlet oxygen when encapsulated in all systems was investigated. The nature of the surfactant influenced the monomer/aggregates ratio, directly influencing the singlet oxygen quantum yield (Φ_Δ). The Φ_Δ reached the maximum when the PS was incorporated in the triblock copolymer Pluronic® F-127 (Φ_Δ ca. 1) and the lowest values when encapsulated by ionic micelles, suggesting that Pluronic®

F-127 is a promising surfactant to incorporate ZnMintPc. The micelle constituents are also important to modulate its interaction with biological media, particularly the cell membrane and organelles, changing the mechanism of incorporation and cytolocalization of the drug.[92] The cytotoxicity can also be tuned by changing the chemical constituents of the micellar monolayer. Zhiyentayev *et al.*[93] studied the influence of nine Pluronic copolymers on the phototoxicity of chlorin e6 (Ce6), demonstrating that it increases as function of the copolymer molecular mass, and that only hydrophilic Pluronics (F-127, F-108, F-68 and F-87) were effective at nontoxic concentrations.

Pluronics are among the main block copolymers investigated to produce micellar systems. Photofrin® II (Photomedica Inc.), a commercially available PS, was encapsulated in polymeric micelles prepared using a mixture of Pluronic® P-123 and F-127 and the photodynamic activity evaluated using two cancer model cell lines, breast MCF-7/WT (caspase-3 deficient) and ovarian SKOV-3 (resistant to chemotherapy). The micellar nanosystem presented higher biocompatibility with lower cytotoxicity in the dark, and increased ROS level and enhanced PDT activity against tumor cells, as compared to free Photofrin® II.[94]

Yang *et al.*[95] compared the photodynamic efficacy of hematoporphyrin (Hp) incorporated in different nanosystems (liposomes, micelles and polymeric nanoparticles) with similar size (112 to 135 nm). All nanoformulations were more effective in reducing cell viability of human lung epithelial carcinoma A549 cells as compared to Hp in solution. Among them, the micellar formulation constituted by Pluronic L122 block copolymers presented the highest cellular uptake and photodynamic activity.

4.1.3. PEGylated liposomes and micelles

Although liposomes and micelles have been extensively used in PDT in recent years due to their capacity to incorporate hydrophobic and hydrophilic photosensitizers, and their ability to accumulate in tumor tissues through the EPR mechanism, they are still susceptible to recognition by hosts' immune system and rapid uptake by the reticuloendothelial system (RES).[96] In this context, PEG is a water-soluble and biocompatible polymer with great acceptance for clinical applications due its low toxicity, non-immunogenicity and antigenicity, in addition to the high tolerance by protein.[4] Thus, among the alternatives to solve this specific problem, the most well established strategy to protect them from being recognized by opsonins and taken up by the RES is grafting PEG on their surface.[67]

Bovis *et al.*[96] investigated the biodistribution and accumulation of two PEGylated liposomal *m*-THPC formulations (FosPEG 2% and FosPEG 8%) increasing the blood plasma circulation and EPR effect, enhancing tumor selectivity in comparison to Foscan®. The antitumor efficiency of ZnPc encapsulated in similar polyethylene glycol coated liposomes was investigated using human extrahepatic cholangiocarcinoma (Sk-Cha1) cells as model.[97] The lipid concentration was used to modulate the extent of particles uptake and cell death induced by PDT-mediated oxidative processes. Oliveira *et al.*[98] showed that the presence of cholesterol improves the stability and photodynamic activity of ZnPc incorporated in unilamellar liposomal formulations, by optimizing the release and modulating its phototoxicity against several human tumor cells.

Two interesting systems based on *p*-THPP were prepared and evaluated by Nawalany *et al.*:[99] (*i*) by incorporating in sterically stabilized liposomes, and (*ii*) by attaching PEG to the porphyrin ring (*p*-THPP-PEG2000). Both liposomal formulations presented lower cytotoxicity in the dark and higher photodynamic activity than free *p*-THPP.

PEG is also frequently used for preparation of polymeric micellar systems. In fact, high loading efficiency and loading density were reported for hydrophobic *m*-THPP in polyethylene glycol-co-poly(D,L-lactic acid) (PEG-PLA) micelles, generating a formulation that exhibits low dark toxicity (less than 10%) and higher than 90% phototoxicity against human head and neck cancer cells *in vitro* after incubation and irradiation.[100] The same block copolymer PEG-PLA was used to incorporate PPIX. Photophysical studies showed the formation of PPIX aggregates inside micelles when high concentrations (4%) of PS were incorporated as evidenced by the decrease of $^1O_2$ quantum yields. However, PDT efficacy in cancer cell models showed an opposite trend and was higher with a 4% PPIX formulation, as a result of the larger amounts of porphyrin delivered to cells.[101] PPIX was also incorporated in pH-responsive block copolymer micelles in order to enable simultaneously tumor diagnosis and therapy *in vivo*. This micellar system is based on the combination of hydrophilic methoxy-polyethylene glycol (mPEG) with poly(β-amino ester) and presents pH-responsive micellization/demicellization transition at acidic pH conditions prevalent inside tumors, enabling the release of large enough concentrations of PPIX to allow clear fluorescent imaging of tumors and their complete ablation after irradiation with red light, whereas free PPIX led to incomplete cell death.[102]

The silicon phthalocyanine Pc4 is a highly hydrophobic second-generation photosensitizer that has showed promising results for PDT treatment of cancer. In order

to allow its compatibility with aqueous media, it was incorporated in less than 100 nm polymeric nano-micelles based on polyethylene glycol-block-poly-ε-caprolactone (PEG-PCL) copolymer, with an encapsulation efficiency of 70%. Significant phototoxicity and cell death, probably by apoptosis, was reported with MCF-7c3 human breast cancer cells *in vitro*.[103]

4.1.4. Targeting properties

Strategies based on the control of the lipidic and micellar layer composition and binding of targeting molecules, such as antibodies, aptamers and peptides on their surface, have been investigated[71] to increase the selectivity of liposomal and micellar formulations.

A liposomal formulation for specific delivery of ZnPc to the cytoplasmic membrane was developed by Kim *et al.*[104] using 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] and 1,2-dioleoyl-3-trimethylammonium-propane. This formulation increased ZnPc accumulation in tumor cells membrane, inducing cell necrosis due to enhanced local ROS generation and phototoxicity.

Folic acid (FA) is one of the most used biomolecules to develop nanoparticles with higher selectivity towards tumor cells. The folate receptor (FR) is over-expressed and is being explored to enhance the selectivity of drug delivery systems to many types of human cancer cells. *m*-THPC was incorporated in FA-functionalized PEGylated liposomes to develop a tumor selective nanosystem for PDT application. *In vitro* results obtained with KB cells showed that FA-liposomes doubled the uptake of *m*-THPC as compared to conventional liposomes and enhanced the photo-induced cytotoxicity.[105]

Folic acid was used to functionalize micelles made of polyvinylpyrrolidone (PVP) containing ZnPc as PS. Zinc phthalocyanine molecules are accommodated in polymer micelles in the monomeric state as confirmed by UV-Vis spectroscopy, greatly enhancing the efficiency of singlet oxygen production and the photodynamic activity *in vitro* and *in vivo*.[106]

The introduction of molecules capable of binding appropriate receptors, that are overexpressed by tumor cells or at the tumor vasculature or surface, have also been explored to improve the selectivity of micellar systems used in PDT.[107] Chen *et al.*[108] described a very low dark toxicity and significantly higher phototoxicity of a porphyrin incorporated in micelles based on a surfactant-like tetra-tail amphiphilic peptide $[(C_{18})_2K]_2KR_8GRGDS$, consisting of four hydrophobic aliphatic tails and a hydrophilic peptide head group. This amphiphilic peptide has an arginine-glycine-aspartic acid (RGD) sequence, which confers selectivity for tumor cells and was responsible for the enhanced uptake by HeLa and 293T cells as confirmed by laser-scanning confocal microscopy. Master *et al.*[109] reported the encapsulation of Pc4 in polymeric micelles grafted with GE11-peptides to enhance the selectivity towards head and neck cancer cells. The nanoformulation presented faster and higher uptake by tumor cells, which resulted in higher phototoxicity *in vitro* and *in vivo*.

On the other hand, Paszko *et al.*[110] showed that the conjugation of transferrin to liposomes loaded with Foscan® did not increase the photodynamic efficiency against OE21 esophageal cancer cell line, as compared to the liposomal formulation prepared without transferrin or even the non-encapsulated PS.

Syu *et al.*[111] designed folate-conjugated polymeric micelles, with diameter about 100 nm, and able to accumulate in tumor cells targeting the folate receptors overexpressed in KB cells, for *m*-THPC delivery. No significant adverse effects were observed in *in vivo* mice models, and after an extended delivery time, a single dose of folate-conjugated *m*-THPC loaded micelles showed higher antitumor effects (tumor growth inhibition of 92%) than free *m*-THPC, inhibiting cell proliferation and reducing the density of blood vessels. In addition, the folate-conjugated delivery system decreased the skin phototoxicity and reduced to a third of the usual photosensitizer dosage necessary to achieve similar antitumor efficacy using the free PS.

The cellular and subcellular targeting strategy was explored by Xu *et al.*[112] to enhance the PDT efficacy. A cationic porphyrin derivative (MitoTPP) was synthesized as the mitochondrion targeting PS, and encapsulated into the acid responsive and FA-modified polymer micelles. This nanosystem was preferentially uptake by FR-positive cancer cells releasing in the cytoplasm MitoTPP molecules that selectively accumulate in mitochondria, as shown by confocal microscopy analyses, causing oxidative damage and apoptosis upon irradiation.

4.1.5. Topical PDT

Liposomes are considered excellent systems to carry and delivery drugs because they are able to incorporate both hydrophobic and hydrophilic compounds, allowing the development of stable formulations.[113] In fact, some hydrophilic PSs applied for topical PDT present low skin penetration, but this problem can be solved by their incorporation in liposomes, thus greatly enhancing PDT activity. For example, RB is a potent hydrophilic photosensitizer based on xanthene chromophore that has largely been overlooked for PDT application because of its low lipid solubility and low capacity to cross

biological barriers such as cell membranes. However, a multivesicular RB liposomal formulation, constituted by D,L-dipalmitoyl-phosphatidyl choline, cholesterol and tripalmitin, was able to cross the epidermis and reach the dermal layers after topical application to mice skin, whereas free RB accumulated in the epidermis.[114] ALA also is a hydrophilic molecule that presents limited capacity to cross the skin and cell membranes. Thus, several approaches have been investigated to enhance ALA delivery, such as the development of new more lipophilic synthetic ALA derivatives (for example MAL), or its entrapment in more lipophilic vehicles, such as liposomes.[115] In fact, liposomes prepared with dipalmitoyl-phosphatidyl choline were shown to increase the uptake of ALA by human cholangiocarcinoma HuCC-T1 cells, enhancing the photodynamic effects.[116]

Sutoris *et al.*[117] reported that a liposomal formulation containing hydroxy-aluminum phthalocyanine (AlOH-PC) cured 100% of experimental animals evaluated for topical PDT treatment of prostate carcinoma. The same group described the application of this formulation for topical treatment of mammary carcinoma, leading to complete tumor remission in 90% (9 in 10) of experimental animals, whereas the commercially available Metvix® only postponed the tumor growth.[118] Temizel *et al.*[119] evaluated the PDT efficacy against HeLa and AGS cancer cell lines of a liposomal formulation of PPIX functionalized with lipophilic oleylamine side-arms (PPIX-Ole) and with 1,2 dioleyl-sn-glycero-phosphatidylcholine (PPIX-DOPC). Both formulations were more photoactive than PPIX in solution, where the degree of toxicity is dependent on the liposomal concentration and type of cancer cell.

In a recent report, squamous cell carcinoma in mice model was successfully treated with liposomes decorated with ICG-C18, a more hydrophobic derivative of indocyanine green (ICG). ICG is highly fluorescent (λ_{em} ca. 820 nm), has low toxicity and generates heat and singlet oxygen when irradiated with NIR (800 nm) light. Because of these properties, it has been used for optical imaging and as PS in PDT. ICG-C18 incorporated in liposomes was shown to be biocompatible and phototoxic, inducing apoptosis in squamous cell carcinoma murine model *in vitro* and *in vivo*.[120] Shemesh *et al.*[121] also demonstrated that indocyanine green loaded liposomes are effective in inhibiting triple negative breast cancer cells growth after PDT treatment *in vitro*.

4.1.6. Combined therapy

As mentioned before, liposomes, micelles and others nanosystems can also be employed to incorporate chemotherapeutic agents, and the combination of chemoterapy and PDT is shown to be an interesting alternative to be explored. Recently,[122] Dox) and Ce6 co-encapsulated in PEGylated liposomes was shown to be released upon irradiation, thanks to the photodynamic action of PS on the liposomal membrane, accumulating in the nuclei, enhancing the *in vitro* and *in vivo* cytotoxic effects as compared to liposomes containing Dox or Ce6 separately.

Park *et al.*[123] developed amphiphilic Ce6 conjugated to Pluronic® F-127 self-assembling units capable to form 30 nm diameter micelles that were loaded with Dox. *In vitro* and *in vivo* assays carried out on drug-resistant cancer cells demonstrated that $^1O_2$ causes cell membrane damage (lipid peroxidation) enhancing the cellular uptake of Dox, thus overcoming the drug resistance without undesirable side effects. Similar strategy was used by Saravanakumar *et al.*,[124] who designed and explored a novel micellar system based on the biocompatible and visible-light responsive amphiphilic copolymer PEG-PCL. Ce6 and the hydrophobic anticancer drug Dox were co-encapsulated and the potential of this micellar formulation investigated as a dual-drug carrier for enhanced photodynamic therapy. The double bond of vinyldithioether, the linker unit in between PEG and PCL, can readily be cleaved by singlet oxygen since they react forming the unstable dioxetane intermediate. So, after irradiation, the generated $^1O_2$ disrupt the polymeric linker releasing the monomeric PS and Dox in high enough concentration to induce synergistic anticancer effects. Yu *et al.*[125] explored a similar strategy encapsulating the chemotherapic agent 5-fluorouracil (5FU) in novel core-shell cross-linked dextran-hemin micelles. Hemin was used as both, the phototrigger for the controlled release of 5FU and as the PS responsible for photodynamic activity.

Therapies other than chemotherapy have also been combined with PDT. Pluronic® F-68 was used by Chu *et al.*[126] to encapsulate natural chlorophyll (Chl) extracted from plants, in order to generate a micellar system for cancer imaging and therapy. It accumulated in mouse tumor tissues and inhibited its growth after submitted to an irradiation protocol combining laser-triggered photothermal (PTT) and photodynamic effects. A synergic anti-tumor effect of PTT and PDT was observed *in vitro* and *in vivo* assays by Gong *et al.*[127] using a polymer based theranostic platform made of poly(maleic anhydride-alt-1-octadecene) grafted with a long PEG-amine (5 kDa) and a short diamine-PEG (324 Da) (C18PMH-PEG 5k/PEG 324 -NH2), which was then conjugated with the photosensitizer Ce6 complexed with Ga^{3+}. This system, denominated C18PMH-PEG-Ce6, was utilized to encapsulate the photothermal agent IR825 generating IR825@C18PMH-PEG-Ce6

micelles containing both agents. The combined treatment *in vivo* damaged both the surface and the interior of tumors under mild doses of light. In fact, 7.2 J cm^{-2} for PDT and 108 J cm^{-2} for PTT are rather low doses as compared to those generally used in PDT or PTT applications.

4.2. Polymeric particles

Polymer based nanoparticles (NPs) are considered more attractive DDS than liposomes and micelles due to their higher stability, small diameter and sharp particle size distribution, which contributes to their passive targeting delivery via the EPR effect. Also, the small size prevents their recognition by macrophages and proteins, thus allowing prolonged circulation time in the blood.[6] Furthermore, a larger amount of drug can be incorporated in them with a higher control on the release process.[67] In fact, biologically active substances can be dissolved or dispersed in, or coated or encapsulated by the polymeric matrix, protecting the drug from harsh environments, such as pH, light and enzyme induced degradation processes. Polymeric NPs are classified as nanospheres and nanocapsules (Figure 3) presenting less than 1000 nm diameter. Nanocapsules are core@shell systems formed by a polymeric shell disposed around a hydrophilic or lipophilic core. The drug can be dissolved/dispersed in the core and/or in the polymeric shell. In contrast, nanospheres are solid polymeric structures in which the therapeutic molecules can be entrapped or adsorbed.[128] Figure 3 shows an illustration of a polymeric nanocapsule with a lipophilic core and of a polymeric nanosphere where hydrophobic PSs can be dissolved/adsorbed. Adequate modifications on nanocapsules and nanospheres polymer composition can allow the incorporation of hydrophilic and amphiphilic PSs.

4.2.1. Poly(lactic-co-glycolic acid) - PLGA nanoparticles

All components of polymeric nanosystems should have good biocompatibility generating biodegradable NPs. Among them, poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymer (PLGA) have been extensively studied because they have already been approved for clinical use by the U. S. Food and Drug Administration (FDA). Their degradation under physiologic conditions releases lactic and/or glycolic acid that are easily metabolized and eliminated by the organism.[129] PLGA were first approved in 1989 and is being widely employed, since then on the design of DDS because of the good stability, reliability and fine control achieved in the release of drugs over periods ranging from days to weeks.[130]

The effectiveness of a NP formulation of *meso*-tetra(*p*-hydroxyphenyl) porphyrin (*p*-THPP) based on PLGA was evaluated using EMT-6 tumor cells. An enhanced PS accumulation and increased cell death rate upon irradiation was reported as compared to free *p*-THPP.[131] The cytotoxicity and intracellular accumulation of analogous nanosystems but loaded with *m*-THPC was investigated by Low *et al.*[132] using human colon carcinoma cells (HT29) as model. A significant reduction of dark cytotoxicity, one of the main limitations of this PS, was observed for the *m*-THPC incorporated in PLGA and in PEG/poly-(d,l-lactide-co-glycolide) (PEG-PLGA) nanoparticles. However, the presence of PEG in the NPs accelerated the PS release and reduced the cellular uptake, decreasing the PDT activity to that of free *m*-THPC level.[133]

Silveira *et al.*[134] showed that three different photosensitizers, (5,10,15,20-tetra(3-hydroxyphenyl)porphyrin, 5-hexyl-10,20-bis(3-hydroxyphenyl)porphyrin and 5-hexyl-10,15,20-tris(3-hydroxyphenyl)porphyrin), with different amphiphilic properties but similar singlet oxygen quantum yields, encapsulate in the same nanosystem (PLGA nanoparticles) exhibit the same cytotoxicity and cytolocalization in the perinuclear region of the cells. This fact indicated that the interaction and incorporation of encapsulated PSs by cells are mainly governed by the characteristics of capsules rather than PSs' interaction properties.

PEGylated PEG-PLA NPs were also employed to encapsulate indium(III) phthalocyanine (InPc), a hydrophobic PS that aggregates in high polarity media, thus hindering their systemic administration and restricting clinical studies. Souto *et al.*[135] evaluated the PDT efficacy of NPs loaded with InPc and their cellular uptake using MCF-7 breast tumor cells. They showed that factors, such as the PS concentration, incubation time and laser power also influence the photodynamic effects. In general, InPc incorporated in PEG-PLGA nanoparticles were more efficient in reducing MCF-7 cell viability than the free PS. For example, for a light dose of 7.5 J cm^{-2} and laser power of 100 mW, encapsulated InPc reduced the viability to 34 ± 3% whereas the PS in the free form reduced only to 60 ± 7%.

The incorporation of photosensitizers in polymeric NPs also considerably improves the photodynamic efficacy, due to the delivery of increased concentrations of phototherapeutic agents to the target tissues. Konan-Kouakou *et al.*[136] reported a seven times increase on *in vitro* and *in vivo* phototoxicity and enhanced photodynamic efficiency of verteporfin when encapsulated in PLGA, due to increased uptake rate of the drug by cultured tumor cells, allowing better control of tumor growth. Silva *et al.*[137] showed for the first time that PLGA-based NPs are able to increase skin penetration, enhancing *in vitro* retention of PPIX in

both stratum corneum and epidermis + dermis, which is very promising for topical delivery of PSs to skin cancer and other skin diseases for PDT treatment. Fadel *et al.*[138] studied the influence of ultra-sonication time on PLGA nanoparticles characteristics and observed that it did not affect the encapsulation efficiency of ZnPc, but the particle size was significantly decreased as a function of the processing time. The histopathological examination of animals after PDT treatment with this polymeric formulation showed a significant improvement and regression of tumor cells. According to Shi *et al.*,[139] PLGA NPs formulations can enhance the delivery and the production of PPIX in human skin squamous carcinoma cells *in vitro*, providing a promising strategy for topical PDT treatment based in ALA. Wang *et al.*[140] reported similar results very recently.

4.2.2. Other polymeric nanoparticles

Chitosan and its derivatives have unique properties, such as high hydrophilicity, biocompatibility and biodegradability, and are suitable for preparation of nanocarriers responsive to external and/or internal physical and chemical stimuli, such as pH, light and temperature, that can be explored in targeted drug delivery.[141] Recently, Graciano *et al.*[142] evaluated the efficacy of chitosan particles to incorporate and deliver toluidine blue O (TBO) to buccal tissues, as well as their PDT activity. The chitosan formulation was shown to enhance TBO retention and induce cell apoptosis *in vivo* after laser irradiation.

Some authors have assigned the contrasting cell uptake and photodynamic activity of a given PS to the methodology employed for incorporation in the polymeric NPs. For example, glycol chitosan NPs modified with hydrophobic groups were shown to be suitable for physical incorporation of hydrophobic Ce6 photosensitizer[143] generating Ce6-loaded glycol chitosan nanoparticles (HGC-Ce6). Alternatively, amphiphilic HGC-Ce6 conjugates, prepared by bonding Ce6 molecules to glycol chitosan polymers, were used to self-assemble NPs in aqueous media (GC-Ce6). Both, HGC-Ce6 and GC-Ce6, presented similar average diameters (300 to 350 nm), were rapidly uptaken by cells and exhibited similar *in vitro* singlet oxygen generation quantum yields. However, GC-Ce6 showed longer circulation time and accumulated more efficiently in tumor cells than the HGC-Ce6 formulation, resulting in higher therapeutic efficacy *in vivo*.

Baumann *et al.*[144] encapsulated a Rose bengal-bovine serum albumin (RB-BSA) conjugate using two types of amphiphilic block copolymers (poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline), PMOXA-PDMS-PMOXA; and poly(*N*-vinylpyrrolidone)-block-poly-(dimethylsiloxane)-block-poly(*N*-vinylpyrrolidone), PNVP-PDMS-PNVP; and showed that their molecular properties can influence particles characteristics, such as size, stability, encapsulation efficiency and uptake by cells. Nanoparticles based on PMOXA-PDMS-PMOXA presented higher RB-BSA encapsulation efficiency and stability, are rapidly uptake by HeLa cells and produce ROS in high enough concentration for PDT application upon illumination.

PEG modified gelatin NPs (HB-PEG-GNP) loaded with HB induced mitochondrial damage after irradiation with light leading to cell apoptosis *in vitro* and *in vivo*. Experiments using mice bearing a solid tumor showed a significant regression rate of ($38.5 \pm 2.2\%$, $p < 0.05$) in contrast to ($29.36 \pm 1.62\%$) when treated with HB-PEG-GNP and free HB, respectively.[145] Biodegradable NPs based on HB encapsulated in polyethylene glycol modified gelatin (PEG-GEL) and polylactic acid (PLA) particles was efficiently internalized by MCF-7 human breast adenocarcinoma, AGS human gastric sarcoma, and mice specific DLA Dalton's lymphoma tumor cells, presenting enhanced PDT activity as compared to the free photosensitizer.[146]

Core/shell polymeric NPs (nanocapsules) also present promising results for encapsulation of many drugs and photoactive substances. Stable oil cored 200 nm diameter nanocapsules based on 1.75% (m/v) soybean lecithin, 1.25% (m/v) Poloxamer 188, 2.5% (v/v) soybean oil, and 0.75% (m/v) PLGA polymer, and loaded with chloroaluminum phthalocyanine (ClAlPc), were developed by Siqueira-Moura *et al.*[147] An encapsulation efficiency of 70% was achieved without affecting the photochemical properties of the PS as attested by the high singlet oxygen quantum yields and significant photodynamic activity even upon irradiation with low light doses.

Poly(*n*-butylcyanoacrylate) (PBCA) nanocapsules stabilized by non-ionic aldonamide-type surfactants were prepared by Wilk *et al.*[148] based on oil in water (o/w) microemulsion method and used to incorporate the hydrophobic PS cyanine IR-780. Four different surfactants, namely dicephalic *N*-dodecyl-*N*, *N*-bis[(3-D-glucoheptonylamido)propyl]amine (C12DGHA) and *N*-dodecyl-*N*,*N*-bis[(3-D-gluconylamido)propyl]amine (C12DGA), as well as gemini *N*,*N*'-bisdodecyl-*N*,*N*'-bis[(3-glucoheptonylamido)propyl]ethylenediamine (bis(C12GHA)) and *N*,*N*'-bisdodecyl-*N*,*N*'-bis[(3-gluconylamido)propyl]ethylenediamine (bis(C12GA)), were synthesized. *In vitro* assays carried out with breast cancer cells revealed that PBCA NPs are biocompatible and the biological responses to PDT treatment depend on the surfactant structure. In fact, this parameter seems to modulate particle uptake by cells.

4.2.3. Effects on physicochemical properties, cytolocalization and PDT efficacy

Although polymeric particles enable the incorporation of both, hydrophilic and hydrophobic molecules, they are mainly used to disperse lipophilic compounds inside, improving their biocompatibility, stability and bioavailability. Liu *et al.*[149] devised two polymeric nanosystems to encapsulate the hydrophobic PS silicon phthalocyanine dichloride ($SiPcCl_2$). Poly(*N*-isopropylacrylamide) (pNIPAM) microgel particles, decorated or not with lipid molecules (Pc@pNIPAM/lipid and Pc@pNIPAM, respectively), were prepared to improve the biocompatibility of $SiPcCl_2$ and prevent its aggregation in aqueous media. The phase transition of pNIPAM was explored to release that hydrophobic PS from both nanosystems inside HeLa cells by a thermo-triggered mechanism, improving the confocal fluorescence imaging and the PDT effect. The incorporation of lipid molecules improved significantly the encapsulation efficiency of $SiPcCl_2$.

Hypocrellin A (HA) is a perylenequinoid pigment isolated from a traditional chinese medicinal fungus, which exhibits excellent antiviral and antitumor properties. However, its hydrophobicity, photodegradation and dark cytotoxicity limits its clinical application. However, Qi *et al.*[150] demonstrated that the incorporation of HA in polymeric particles is an excellent alternative to overcome those limitations, as confirmed by the high dispersibility in aqueous media and the enhanced photostability measured spectrophotometrically. Further *in vitro* experiments demonstrated that HA loaded PLGA NPs were taken up by A549 tumor cells and exhibited reduced dark cytotoxicity, while maintaining excellent anti-tumor properties and ROS production ability.

Indocyanine green (ICG) is another promising photosensitizer, whose instability in aqueous solution is been overcome by incorporation in biocompatible nanosystems. For example, its encapsulation in a biologically inert polymeric matrix improved stability and photoactivity against human breast adenocarcinoma cells (MCF-7) and hepatocellular carcinoma cells (HepG2).[151] PCL nanoparticles with a mean diameter of 187 nm, prepared by a solvent emulsification-evaporation method, were used to incorporate ZnPc for *in vitro* PDT of human lung adenocarcinoma A549 cells. The cellular viability determined after 24 h of incubation showed that ZnPc-loaded NPs and free photosensitizer eliminated about 95.9 ± 1.8% and 28.7 ± 2.2% of A549 cells, respectively, after irradiation with red light. The results also showed that the phototoxicity was time dependent until up to 4 h and concentration dependent at 0-5 μg of ZnPc, and cells viability decreased as a function of the light dose in the 10-100 J cm^{-2} range.[152]

Oil-cored PBCA nanocapsules with unimodal size distribution and spherical shape were prepared by interfacial polymerization[153] with a quite high encapsulation efficiency (91.7%) of cyanine IR-768. This nanocarrier efficiently delivered the PS *in vitro* to Dox-sensitive and Dox-resistant MCF-7 cell lines, promoting a significant decrease in cell viability after irradiation. In addition, low hemolytic and toxic effects were reported in the dark.

Another interesting effect pursued by encapsulation is the control of cytolocalization since this parameter is fundamental for PDT activity as well as to avoid side effects. In this context, micro- and nanocapsules made of marine atelocollagen and xanthan gum were demonstrated to be excellent vehicles for lipophilic porphyrins and metalloporphyrins. In fact, carefully designed studies were performed, demonstrating that they act as shuttles penetrating and crossing the tumor cell membrane (HeLa cells), releasing the photosensitizer in the cytoplasm, as illustrates in Figure 4a.[49,154] Interestingly, no significant changes could be observed in the cell structure after irradiation, showing that just enough damage was caused in internal organelles, such as mitochondria and lysosomes, to trigger apoptosis, the controlled death mechanism. In contrast, the respective free porphyrin photosensitizer was mainly concentrated in the cell membrane (Figure 4b), promoting severe damage as demonstrated by trypan blue and propidium iodide assay, inducing necrosis.[155]

4.2.4. Targeting properties and combined therapies

One of the main advantages of polymeric NPs is the easy surface modification with functional molecules and biomolecules, such as antibodies, in order to improve selectivity.[4] For example, Abdelghany *et al.*[156] conjugated an antibody targeting the death receptor 5 (DR5), a cell surface apoptosis-inducing receptor up-regulated in various types of cancer cells, on the surface of chitosan/alginate NPs loaded with hydrophilic *meso*-tetra(4-*N*-methyl-pyridynium)porphyrin (TMPyP) tetra-tosylate as photosensitizer agent. The presence of the antibody enhanced the uptake and therapeutic effect of TMPyP on HCT116 cells.

PLGA NPs with dual surface modification, PEG and FA, were designed and prepared by Ma *et al.*[157] to enhance the accumulation of ICG in tumor tissues. Biodistribution studies performed with mice models, xenografted with MDA-MB-231 human breast cancer cells with high expression of FR, indicated longer circulation time in blood and higher ICG concentrations in plasma and tumor in detriment of liver as compared to non-modified PLGA nanoparticles.

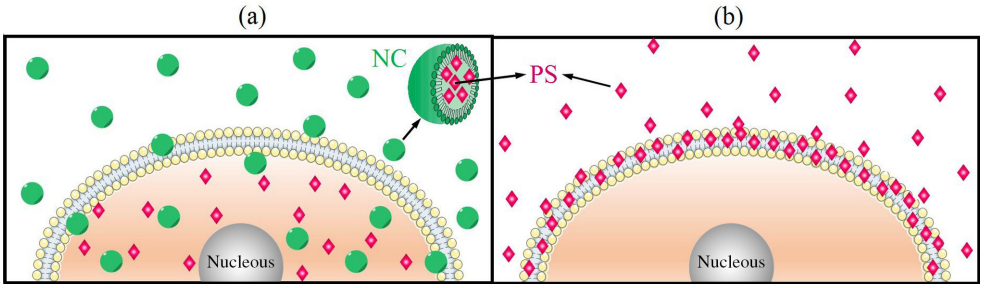


Figure 4. Illustrations of the differences on cytolocalization of a lipophilic photosensitizer (PS), a porphyrin derivative, in tumoral cells. Photosensitizer (a) encapsulated in polymeric nanocapsules (NC) and (b) non-encapsulated (free porphyrin).

Cancer therapies exploiting either additive or synergistic effects arising from the combined action of two or more biologically active species can maximize the therapeutic efficacy. Conte *et al.*[158] described a multifunctional polymeric nanocarrier based on ZnPc and the chemotherapic agent docetaxel (DTX). Core-shell NPs were prepared using the biodegradable and amphiphilic block-copolymers PCL (B) and poly-ethylene oxide (PEO, A), forming AB and ABA architectures. The ZnPc/DTX-loaded system showed higher cytotoxicity *in vitro* as compared to NPs loaded only with DTX, thus demonstrating the advantage of combining the antitumor activity of both, DTX and ZnPc.

Methylene blue is a promising photosensitizer that tends to concentrate mainly in endolysosomal vesicles, but a significant nuclear localization was observed, when encapsulated in AOT-alginate nanoparticles[159] and incubated with MCF-7 and 4T1 tumor cell lines. In addition, an enhanced intracellular ROS production and consequent higher phototoxicity was observed inducing the necrosis of those tumor cells. This result was further improved by combining the chemotherapeutic agent Dox. An improved intracellular and nuclear delivery was accomplished for the two drugs, leading to higher ROS production as compared to single drug treatments.[30] The combined action of chemotherapy and PDT was able to overcome resistance mechanisms and improve cytotoxicity towards drug-resistant tumor cells.[160] An increased production of ROS under both, normoxic and hypoxic conditions, was shown to be controlled by the degree of interaction of the cationic photosensitizer with the anionic alginate polymer, enabling efficient electron transfer reactions directly from the excited PS and PDT activity by type I mechanism.

Polymeric nanoparticles are also promising platforms for theranostics, i.e., materials enabling simultaneously the diagnosis and the treatment of cancer. An interesting example is the development of a multifunctional nanocarrier based on biodegradable polyacrylamide NPs by Wang *et al.*,[161] allowing cancer diagnosis by fluorescence imaging and the treatment by PDT just by controlling the dose and wavelength of incident light. The nanoparticles have 44 nm average diameter and PEG and tumor targeting molecules grafted on the surface. A good selectivity was achieved *in vitro* as confirmed by a strong fluorescence from inside tumor cells, and a significant and selective PDT activity after incubation with that nanoformulation.

4.3. Other nanosystems

Although liposomes, micelles and polymeric nanoparticles are the most commonly explored DDS in PDT, other nanosystems also presented promising results for the development of nano-PSs or combined therapeutic agents. Some general aspects on this subject will be reviewed below.

Dendrimers are highly branched molecules constituted by layers of individual dendrons or wedges, that emanate out symmetrically from a central common core, where the number of concentric layers constitutes the dendrimer generation.[162] Higher generation dendrimers can assume macromolecular dimensions ultimately affording nanostructures possessing cavities between branches and a more or less globular shape as many proteins in biological system, thus, behaving as nanocarriers. Dendrimers' dispersion and interaction properties are mainly defined by the outermost molecular layer, which can be tailored by known conventional chemical reactions. Accordingly, molecules can be easily encapsulated in their interior or chemically attached on their surface or core (Figure 5a), thus been extensively explored to incorporate photosensitizers enhancing PSs' biocompatibility, bioavailability and tumor tissue specificity, increasing the potentiality for PDT application.[163] In fact, several dendrimer architectures incorporating porphyrin and phthalocyanine in the structure have been synthesized in the last years as reviewed by Figueira *et al.*[162]

Carbon-based materials, such as graphenes, carbon nanotubes and fullerenes, are interesting due to their very high surface-to-volume ratio, thermal conductivity, structural rigidity and variety of post-chemical modification possibilities.[164] However, it is important to remember at this

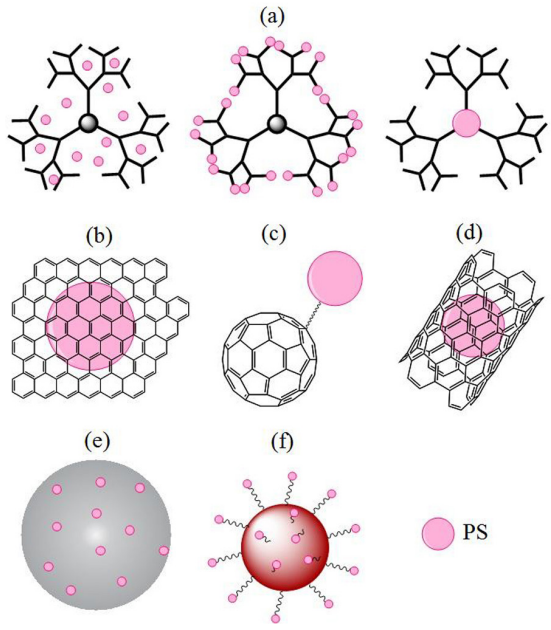


Figure 5. Alternative nanosystems for incorporation/conjugation of photosensitizer (PSs) for photodynamic therapy (PDT) application: (a) dendrimers; (b) graphenes; (c) fullerenes; (d) carbon nanotubes; (e) silica nanoparticles and (f) gold nanoparticles.

point that there are still many concerns about their toxicity, cytotoxicity and clearance properties.

Graphene is a material constituted by a monolayer of sp^2 hybridized carbon atoms bond together in a honeycomb arrangement where the p_z orbitals form an aromatic conjugated π-system.[165] Graphene based nanosystems have been shown to improve the stability, bioavailability, and photodynamic efficiency of photosensitizers. In addition, they present an intrinsic near infrared absorption that can be explored to impart photodynamic and photothermal hyperthermia properties to those nanomaterials for optimum therapeutic activity.[166] Direct physisorption via π-π interaction can be used to load many drugs, particularly hydrophobic PSs (Figure 5b). Recently, Xu *et al.*[167] described the PDT efficacy of a PEG-functionalized and folic acid-conjugated graphene oxide (GO) loaded with a cationic porphyrin derivative, exhibiting preferential accumulation in mitochondria. This nanosystem presented higher phototoxicity toward FR-positive cells and was preferentially uptake by cancer cells overexpressing folate receptors.

Fullerenes are round shaped molecules formed by 60-100 carbon atoms characterized by an extended π-conjugated system exhibiting long-lived excited triplet state capable to generate ROS upon absorption of ultraviolet and visible light.[1] A poly(ethyleneimine) (PEI) functionalized fullerene loaded with Dox (C60-PEI-Dox), prepared by Shi *et al.*[168] showed significantly improved *in vivo* therapeutic efficacy for cancer treatment. This result was attributed to a synergistic effect resulting from the combination of chemotherapy and photodynamic therapy using C60-PEI-Dox nanoparticles.

The association of PSs with fullerenes (Figure 5c) can also result in new compounds with enhanced singlet oxygen generation and tumor cell penetration efficiency, as reviewed by Constantin *et al.*[169] This class of nanomaterials can also be functionalized in order to confer targeting properties as demonstrated by Lim *et al.*[164] Hoechst 33258 was bond to target necrotic tumor cells and hyaluronic acid to target CD44 receptors overexpressed in tumor cells surface. Such Hoechst 33258/hyaluronic acid conjugated fullerene showed significantly increased *in vitro* phototoxicity and *in vivo* tumor inhibition properties as compared to fullerene conjugated only with hyaluronic acid.

Carbon nanotubes (CNTS) have emerged as both anticancer drugs and drug delivery agents because present strong optical absorption in the NIR region, that extends until the UV region, suitable for photothermal therapy of cancer cells, as well as for transport of drugs (Figure 5d).[170] For example, single wall carbon nanotubes (SWCNTs) loaded with Ce6 by noncovalent π-π interactions, and wrapped with chitosan to improve dispersibility in aqueous media and biocompatibility, showed high cellular uptake and PDT activity against HeLa cells *in vitro*.[171]

Silica nanoparticles (SiNPs) are highly porous, structurally and chemically inert materials, not susceptible to swelling and other structural changes as a function of medium conditions, such as pH. Furthermore, there are several methods available to control their size, shape, porosity and to encapsulate a great variety of PSs. The surface modification with specific biomolecules has also been explored to confer targeting properties and improve cellular uptake.[172,173] Teng *et al.*[174] developed a nanocarrier platform for PDT, based on phospholipid-capped, PPIX-loaded and FITC-sensitized mesoporous silica conjugated with FA. This multifunctional nanosystem showed selective accumulation in folic acid receptors over expressed in HeLa cells, exhibiting higher cellular and *in vivo* PDT activity than free PPIX, being able to mitigate nearly 65% of B16F10 tumor cells in inoculated nude mice model.

Photosensitizers can be conjugate to SiNPs surface or encapsulated in the silica matrix pores (Figure 5e), changing their photophysical properties, as recently described by Fashina *et al.*[175,176] Zinc phthalocyanine molecules encapsulated in silica nanoparticle pores showed improved triplet and singlet oxygen quantum yields than those grafted on the surface, probably due to the protection provided by the silica matrix. The possibility of encapsulating and attaching drugs on silica nanoparticles surface allowed the development of multi drug delivery systems, combining chemotherapy and PDT,[177] or the construction of theranostic

platforms. For example, Zhao and co-workers[86] incorporated superparamagnetic Fe_3O_4 NPs (contrast agent) in the silica matrix and conjugated methylene blue on the surface.

Gold nanoparticles (AuNPs) have been explored in biomedical applications for more than 40 years and currently efforts are been mainly focused on the development of nanomaterials for diagnosis, vector transfection, drug and gene delivery, hyperthermia treatment, and as imaging probes.[178] Biomolecules and molecules can be easily attached to AuNPs surface (Figure 5f), making them interesting systems for development of multifunctional materials for theranostics. Meyers *et al.*[179] described a nanosystem based on the conjugation of Pc4 and epidermal growth factor peptide (EGF_{pep}) on the surface, presenting enhanced blood circulation time, selective delivery of the PS to tumor tissues and PDT activity. Hematoporphyrin[180] conjugated with 15 and 45 nm large AuNPs were shown to be more phototoxic *in vitro* than free PS. Interestingly, nanocomposites prepared with 45 nm large AuNPs exhibited higher activity, probably because bigger particles are able to transport larger amounts of porphyrin molecules at once to malignant cells. Yu *et al.*[181] combined MB with AuNPs, exploring the intermolecular interactions between the polystyrene-alt-maleic acid (PSMA) layer and MB, producing a material with improved quantum yield for singlet oxygen generation as compared to free MB. The conjugation of transferrin and MB to the surface resulted in 2-fold enhancement of PDT efficiency and apoptosis of HeLa cells.[181]

The attachment of photosensitizers on superparamagnetic iron oxide nanoparticles (SPIONs) is an alternative that has been extensively explored in the last years to combine more than one therapy for treatment of cancer, such as magnetohyperthermia (MHT) and PDT. MHT is based on the heating of magnetic particles when exposed to alternating current (AC) magnetic fields, thus promoting thermally-induced (temperatures ranging from 41 to 46 °C) direct and indirect cellular effects to the microvasculature, blood flow, energy and oxygen status, such as ischemia (decreased blood perfusion) and vascular occlusion (nutrient and oxygen deficiency in neoplastic cells).[182]

Bolfarini *et al.*[182] explored this strategy, combining PDT and MHT in magnetoliposomes loaded with ZnPc conjugated to cucurbituril (CB) derivatives. PDT was shown to be more effective than MHT in reducing the viability of B16-F10 melanoma cells *in vitro*, but the combined treatment doubled the cellular damage and cell death as compared with PDT alone. More recently, a nanoplatform based on liposomes containing SPIONs in the core and *m*-THPC photosensitizer in the lipid bilayer, developed by di Corato *et al.*,[183] was shown to be capable of total solid-tumor ablation in an *in vivo* rodent model by combined PDT and MHT treatment.

All nanosystems described in this review improved the photodynamic activity of free PSs. However, interesting synergic effects, resulted from the combination with other therapies, thus, generating promising platforms with enhanced efficiency[183,184] and theranostic application,[185-187] enabling a myriad of possibilities for development of nanomaterials and formulations for treatment and diagnostic of cancer.

5. Concluding Remarks and Perspectives

Recent advances have reinforced the idea that nanotechnology can provide valuable tools for the development of new photosensitizer formulations with improved PDT activity, enhancing its potentiality for treatment of cancer. Generally, nanosystems improve the solubility, bioavailability, delivery, biodistribution, selectivity and photoactivity of PSs, reducing the formation of aggregates responsible for decreasing the singlet oxygen quantum yields and, consequently, their PDT efficacy. In addition, DDS could also enhance the concentration of PS in the tumor tissue by passive or active targeting mechanisms, according to its physicochemical characteristics, increasing the photodynamic efficiency and selectivity of the therapy thus reducing side effects such as systemic toxicity and skin photosensitivity.

However, the chemical constitution and physicochemical characteristics of the nanosystems, such as size, morphology, charge and shape of nanoparticles will affect their interaction with biological systems influencing the biocompatibility, pharmacokinetics and also their efficiency. Thus, understand and control all these factors is important to design more efficient and safe DDS. In this respect, polymeric nanocapsules and nanospheres are very promising, because of the great number of biocompatible polymeric materials available, allowing the incorporation of high amounts of drugs and the delivery of adequate concentrations by simple control of the polymeric wall cross-linking and resistance. In addition, the polymeric composition can also be modulate in order to insert selected functional groups to the particle surface, controlling the hydrophobic and hydrophilic characteristics as well as the chemical reactions to bind specific molecules/biomolecules for target delivery.

The toxicity of those nanomaterials should also be addressed in order to assure their bio-safety in clinical protocols. Few were the studies considering the dark toxicity whereas the long-term effects were almost neglected. In fact, the lack or insufficient information

about them seems to reflect the low number of nano-PDT drugs approved so far. Thus, the success of PDT based on nanomaterials and nanoformulations will depend on a concentrate effort involving experts from the several areas (physics, chemistry, biology, biochemistry, pharmacy, medicine, nanomedicine, biomedical engineering, etc.) needed to get well characterized enough materials, as well as understand their interactions with our organism and with light, enhancing the benefit-to-risk ratio to acceptable levels for clinical application. In short, nanotechnology is providing interesting tools for preparation of multi-functional agents, now not only focused in the treatment but also diagnosis of tumors, thanks to the development of efficient targeting mechanisms continuously improving the selectivity of delivery systems, opening the road for theranostics.

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