Nanotechnological approaches to address photosensitizers' limitations: towards improved clinical applicability of photodynamic therapy

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Abstract: Photodynamic therapy (PDT) uses a combination of molecular oxygen, light and a photosensitizer (PS) to generate singlet oxygen or reactive oxygen species (ROS), which can eradicate tumoral cells. All currently approved PSs for cancer treatment are molecular PSs. To date, no nanoparticle-based PSs are used clinically although it has widely been shown that nanotechnology may help to improve the properties of molecular PSs; for instance, molecular PSs suffer from some intrinsic limitations that undermine their therapeutic efficacy. In the present minireview, the most critical weaknesses exhibited by molecular PSs are described, and the potential use of nanoparticles (NPs) to address them and to reach the clinics is discussed.

Keywords: photosensitizer, nanoparticle, photodynamic therapy, cancer, hypoxia.

I. Introduction

Photodynamic therapy (PDT) is currently used to treat some diseases such as acne,[1] macular degeneration[2] and cancer.[3] PDT requires dioxygen, light and a photosensitizer (PS), which is a molecule that can be photoactivated, typically in the visible-to-NIR range. Upon irradiation, singlet oxygen or ROS are produced, which damage cancer cells inducing their death *via* various mechanisms, *viz.* apoptosis, autophagy or necrosis.[4] PDT is a therapy whose origins date back to the early 1900s; though, the first clinical approval of a PS was in Canada in 1993 for bladder cancer treatment.[5] In the last thirty years, this therapy has gained exponential interest since, compared to chemotherapy, it is more selective and therefore reduces significantly side effects. It is indeed well known that chemotherapy can cause undesirable effects to the patient, including vomiting, nausea, tiredness, loss of hair, depression, general pains, etc.[6] PSs present minimal or low toxicity in the dark, but can be activated locally with light, *e.g.* lasers and LEDs, without affecting the whole organism; the light source is placed close to the tumor, which will be affected by singlet oxygen or ROS produced through PS activation.

The efficiency of PDT has progressively been improved through the development of various generations of PSs corresponding to distinct conceptual approaches; hence, a first, second and third generation of PSs have been described so far (**Figure 1**). First-generation PSs were the first to receive clinical approval and include Photofrin® and derivatives of Hematoporphyrin (HpD). These compounds are oligomeric complexes formed by porphyrinic structures linked by alternating ether and ester bonds, which can be excited at about 630 nm. Although they are approved clinically, their therapeutic use is limited due to their complex chemical composition, poor solubility, low selectivity, and weak absorption at 630 nm.[7]

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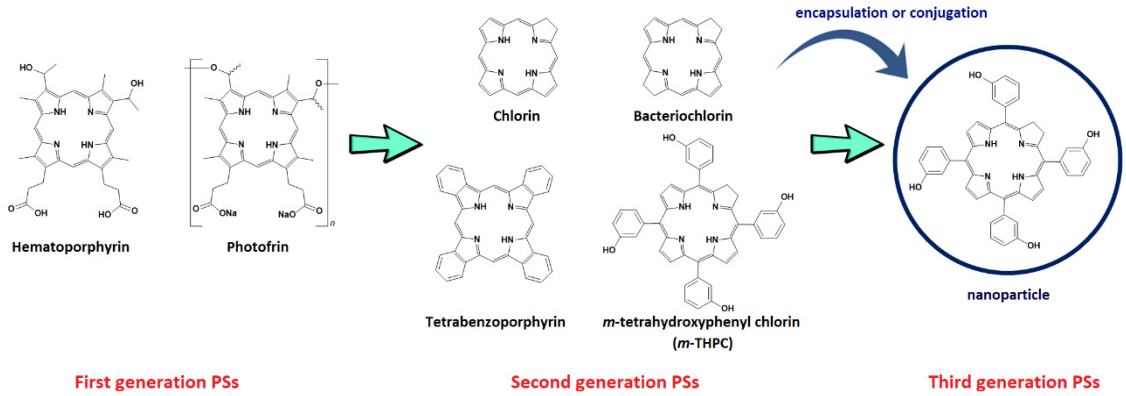

Figure 1. Selected examples of first-, second- and third-generation PSs.

Second-generation PSs are mostly derivatives of porphyrins; they have a well-defined chemical structure and strong absorption in the visible spectrum, which allows obtaining high singlet-oxygen quantum yields. Moreover, better interpretations of dose-response relationships can be obtained as they are not mixtures of oligomeric complexes.[8] Though, second generation PSs also suffer from weaknesses that cannot be solved simply by chemical modification of their structure or by new optimized molecular design. Some of these drawbacks include poor selectivity and water solubility, inefficient targeting, and suboptimal tumor regression. At this point, nanotechnology offers a wide range of possibilities for the so-called third-generation PSs,[9] for instance to improve tumor targeting ability, lower hypoxia resistance, and achieve more effective production of ROS and $^1O_2$.[10]

In the present minireview, the major barriers preventing PDT from reaching its full potential are described. Moreover, the potential of nanotechnology to lower or eliminate these limitations is discussed.

II. Discussion

II.1. Nanomaterials for the improvement of photosensitizers' properties

It is not a simple task to tackle a specific limitation of a PS without affecting other important parameters. For example, the improvement of the water solubility of a PS through conjugation to a water-soluble nanoparticle may alter other properties, such as the cellular uptake, biodistribution, singlet-oxygen quantum yield and so forth. The properties of an ideal PS candidate are now widely agreed upon and are listed below:[11,12]

- good photostability
- long lifetime at the triplet state
- high rate of $^1O_2$ production
- strong absorption in the range of 600-800 nm
- low dark toxicity
- rapid clearance from the body
- low-cost

The characteristics listed above are not sufficient; there are several equally crucial parameters that must be considered:

- tumor targeting and enhanced cellular uptake by cancer cells: high accumulation of the PSs in the tumor mass will minimize healthy-cell damage. High cell internalization will improve the efficacy of the PSs;

- water solubility: low water solubility may lead to the aggregation of the PSs solution, reduction of its bioavailability and diminution of their ability to produce $^1O_2$ / ROS;
- absorption in the NIR (800-1200 nm) to allow the treatment of deep-seated or large tumors: the tissue penetration of light in the range 600-800 nm is about 1 cm, which would only allow treating small-sized and shallow tumors (*e.g.* TLD-1433 against bladder cancer).[13] PSs that can be excited in the NIR range may be used for the treatment of deep-seated tumors as NIR absorption occurs at higher tissue depths;
- production of $^1O_2$ / ROS without the presence of molecular oxygen: most PSs activities are based on the presence of O_2; therefore, their therapeutic effect is lessened in an oxygen-poor environment. The PS-mediated production of cell-damaging (radicalar) species without the consumption of molecular oxygen (or of minimal quantities of it) is essential for the treatment of hypoxic tumors.

Failure to comply with these four features (listed above) will strongly affect the efficacy and clinical transfer of molecular PSs. In that context, the use of nanoparticles (NPs) may help to solve these problems (**Figure 2**).

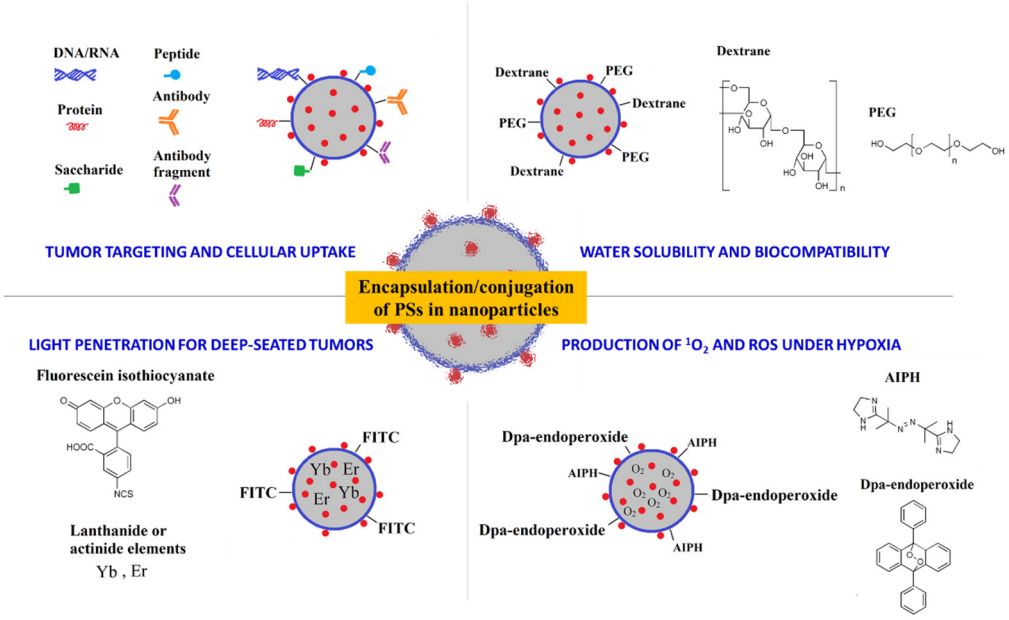

Figure 2. Common PSs' drawbacks that may be overcome with nanotechnological approaches.

II.1.1. Nanoparticles to improve tumor targeting and cellular uptake

Several parameters can be considered when designing new chemotherapeutic drugs like the selectivity, efficacy, or binding affinity. The selectivity of PSs may be tuned by modification of their shape, conformation, flexibility, lipophilic and hydrophilic regions, ability to generate hydrogen bonds, ability to generate electrostatic interactions, and distribution of polarity.[14] It can be pointed out that any structural change(s) of a PS aimed at improving its therapeutic properties may result in a deterioration of its photophysical properties.

The low selectivity of PSs in tumor tissues is a recurrent issue in PDT, even with clinically approved compounds such as Photofrin® and Foscan®. Hence, third-generation PSs with high selectivity have been developed that target cancer cells through their conjugation with tumor-targeting moieties or nanoparticles (**Figure 3**). The improvement of selectivity is usually associated with an enhancement of cancer-cell uptake.

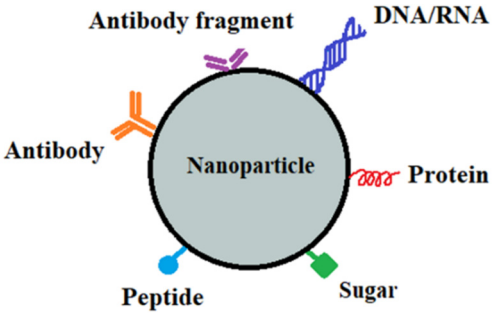

Figure 3. Functionalities that can be grafted onto the surface of a nanoparticle to improve selectivity, cellular uptake, and activity.

Some proteins or receptors are overexpressed on the cell membrane of cancer cells; these may help to increase tumor selectivity and cellular uptake of the drug. Some examples are the folate receptors (FR), transferrin receptors (TfR), integrin receptor (IR) and cell adhesion molecule (CAM) receptors.[15] Moieties to improve both the selectivity and uptake of PSs are very often combined onto the surface of nanoparticles that can enter the cells through endocytosis or phagocytosis.[16] Furthermore, nanoparticles can take advantage not only of active targeting, but also of passive targeting.[17-19] Even not being decorated with targeting moieties, nanoparticles seem to possess an intrinsic ability to accumulate in a higher extent in tumor tissues. Such passive selectivity toward tumors has been long attributed to the so-called enhanced permeability and retention (EPR) effect, due to the easier extravasation of the nanoparticles from leaky tumor vessels.[20-22] However, in the last years it has been demonstrated that the mechanism by which nanoparticles enter solid tumors is more complex and the existence of the EPR effect in human patients is being questioned.[23]

Many nanoparticles exhibiting good passive targeting are polymers that show biocompatibility, biodegradability, high loading capacity and water solubility. For example, Zeisser-Labouèbe *et al.* have reported nanoparticles of polylactic acid, which were loaded with hypericin (Hy), a natural PS used for the photodetection of ovarian micrometastases. These nanoparticles were tested on Fischer 344 rats bearing ovarian tumors and it was shown that they accumulated selectively in the tumor.[24]

However, and unlike active targeting, the selective accumulation of PSs in cancer tissues is not necessarily correlated to their internalization into tumor cells. Due to the high interstitial fluid pressure (IFP) of solid tumors (which according to the Global Cancer Observatory in 2020[25] and the National Cancer Institute in 2021[26] were the most common tumors), a decrease in cellular uptake can occur, this IFP impeding an efficient internalization.[27] The only nanotherapeutic systems that are currently clinically used for chemotherapy and PDT benefit from passive targeting.[28]

Many studies on nanoparticle-assisted delivery of antitumor drugs emphasize the importance of active targeting, which is aimed at increasing effectively the cellular uptake.[29,30] Clemons *et al.* found that functionalization of docetaxel-loaded nanoparticles with the peptide GE11 that actively targets the epidermal growth factor receptor (EGFR) resulted in an improved internalization and cytotoxicity *in vitro*.[31] Schleich *et al.* combined active targeting (using an RGD peptide) and magnetic targeting with paclitaxel-loaded superparamagnetic iron oxide (SPIO) nanoparticles. Enhanced therapeutic effect and eight-fold increase of accumulation compared to passive targeting were hence achieved in tumoral tissues of CT26 mice.[32]

In the case of PSs that produce $^1O_2$ and ROS with very short lifetimes,[33] it is particularly important for their efficacy to favor their cellular uptake through active targeting. Studies on active targeting revealed that the simple conjugation of moieties such as antibodies,[34] antibody fragments,[35] proteins,[36] and peptides[37] with molecular PSs can enrich their properties. Conjugation of PSs to tumor-targeting nanoparticles can significantly improve their propensity to kill cancer cells more selectively. For example, the surface of small gold nanoparticles (4 nm) was functionalized with a zinc–phthalocyanine derivative

(PS), a heterobifunctional polyethylene glycol preventing aggregation, and an anti-HER2 monoclonal antibody for active targeting;[38] these NPs could selectively target breast cancer cells overexpressing HER2 epidermal growth factor receptors and could be internalized through endocytosis (**Figure 4**).

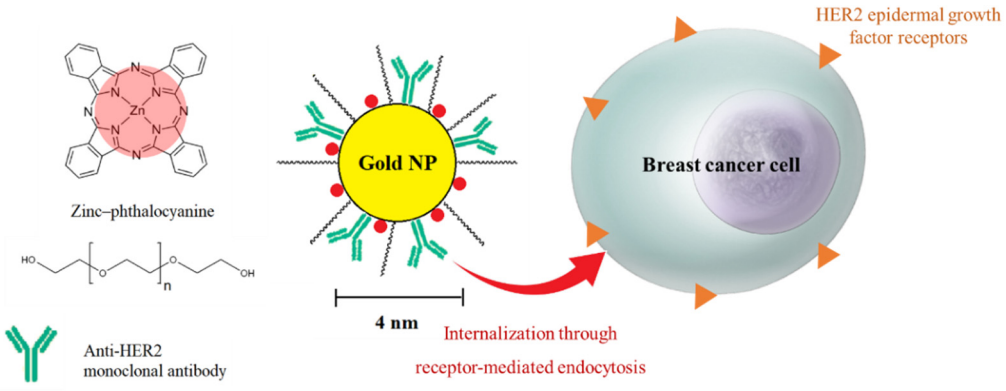

Figure 4. Gold nanoparticles (4 nm) functionalized with a zinc–phthalocyanine derivative (PS), a heterobifunctional polyethylene glycol and an anti-HER2 monoclonal antibody.

Their photocytotoxic properties were evaluated on breast cancer cells overexpressing HER2 antibodies (SK-BR-3), breast cancer cells not overexpressing HER2 (DA-MB-231) and on healthy normal mammary epithelial cells (MCF-10A). The NPs decreased the cell viability by 60% for SK-BR-3 cells, 25% for DA-MB-231 cells and 7% for MCF-10A cells, therefore illustrating the beneficial effect of the functionalized NPs. PEG-liposomes conjugated with transferrin and containing the PS aluminium(III) phthalocyanine tetrasulfonate (AlPcS4) were synthesized and evaluated *in vitro* against HeLa cells. The PEG-liposomes without transferrin were not photocytotoxic, whereas the transferrin-conjugated ones loaded with AlPcS4 were 10 times more photocytotoxic than free AlPcS4, most likely due to their high accumulation in cancer tissues and internalization into tumor cells through endocytosis.[39]

Peptides may be alternative targeting agents. For instance, silk fibroin nanoparticles (SF NPs) were prepared from the protein isolated from *Bombyx mori* cocoons, and were conjugated with the PS chlorin e6 (Ce6) and cRGDyk, a small cyclic peptide that targets the integrin receptor, which is overexpressed on the membrane of tumor cells.[40] Furthermore, the functionalized SF NPs were loaded with 5-fluorouracil (5-FU) to combine photodynamic therapy with chemotherapy. Phototoxicity assays *in vitro* with MGC-803 cancer cells and *in vivo* experiments using male BALB/c-nude mice showed a highly selective accumulation of the multifunctional NPs in the tumoral mass thanks to the cRGDyk peptide, and an excellent reduction of the tumor size upon light irradiation (**Figure 5**).[40]

Since antibodies, proteins and peptides can be very expensive, a cheaper approach consists in using carbohydrates (*viz.* sugars) to target tumor cells. For instance, galactose or sialic acid can be used to respectively target asialoglycoprotein receptors and Siglec receptors, which are overexpressed in liver and pancreatic cells.[41,42] For example, polydopamine nanoparticles conjugated with hyaluronic acid and chlorin e6 were synthesized; these NPs combine photodynamic therapy (Ce6) with photothermal therapy (polydopamine).[43] Thanks to hyaluronic acid, the NPs very efficiently targeted cancer tissues through the CD44 receptor that is overexpressed in many tumor cells.[44] The NPs exhibited high accumulation and internalization *via* endocytosis into cancer cells of HCT-116 tumor-bearing mice, resulting in a potent antitumor effect.

Another promising approach for the selective accumulation of PSs is the use of magnetic nanoparticles to target selectively tumoral tissues. For example, Ni *et al.* developed magnetic nanoparticles whose surface was decorated with tetrakis(4-carboxyphenyl)porphyrin and radiolabeled with ^{89}Zr, the idea being Cerenkov radiation to promote photodynamic therapy without the use of external light.[45] The magnetic

NPS were magnetically guided to the tumor site where they accumulated and significant inhibition of the tumor growth in 4T1 tumor-bearing BALB/c mice was observed.[45]

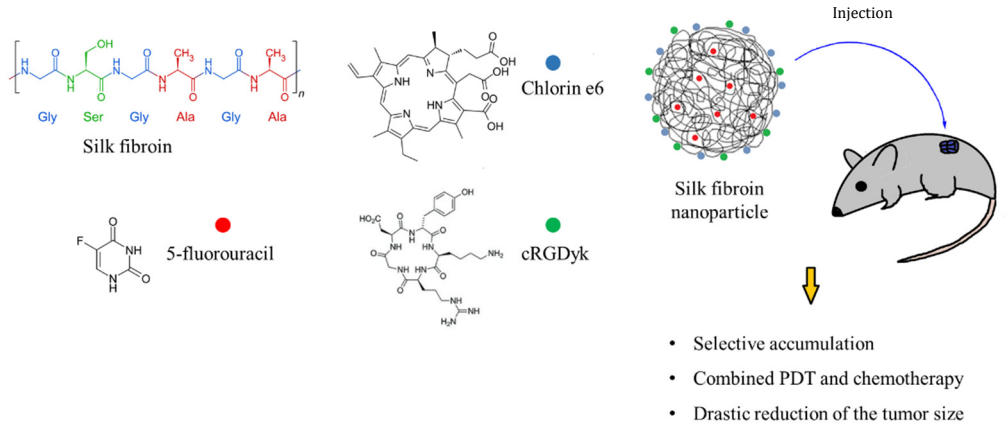

Figure 5. Silk fibroin nanoparticles conjugated with chlorin e6, cRGDyk and loaded with 5-fluorouracil (5-FU) to allow a combination of PDT with chemotherapy.

Magnetic nanoparticles of ~20 nm in diameter functionalized with chlorin e6 were reported by Huang *et al.*[46] The ability of the NPs to target cancer cells *in vivo* was evaluated with nude mice bearing MGC-803 cells (gastric cancer); the data achieved evidenced the ability of the NPs to reach the tumors upon application of an external magnetic field, and a clear regression of the tumor size upon light irradiation.[46]

II.1.2. Nanoparticles to increase water solubility

The poor water solubility of the PSs leads to their aggregation, which mostly affects their bioavailability and light absorption. Other parameters, like biodistribution, targeting, cellular uptake and cytotoxicity, may be altered as well. PSs are very often insoluble in water, even if they are charged, as the result of their usually large size (highly conjugated aromatic molecules with light absorption in the visible range). Low aqueous solubility is a common feature in first-generation PSs and sometimes also in second-generation ones. One common strategy to improve the solubility in water is based on the introduction of hydrophilic groups on the PS structure. For instance, to overcome the low water solubility of the palladium(II)-based PS TOOKAD®, a water-soluble analogue named TOOKAD® Soluble was designed. TOOKAD® Soluble presents a sulfonate group introduced through reaction between TOOKAD® and homotaurine, which leads to the opening of the isocyclic ring by aminolysis; it also presents a potassium carboxylic salt instead of a carboxylic acid group.[47] These chemical modifications make TOOKAD® Soluble a water-soluble PS, which is currently in clinical use for the treatment of low-risk unilateral prostate adenocarcinoma in adult patients in Mexico, Israel and 31 other countries of the EU.[48]

Among the possible functional groups that can be introduced to enhance the hydrophilicity of originally lipophilic molecules,[49] the most used one is the hydroxyl group, especially with drugs.[50-52] The introduction of hydroxyl groups is not always the best solution to increase the aqueous solubility of PSs. For example, the precursor of Hematoporphyrin Derivative (HpD), namely hematoporphyrin, is synthesized by converting the two vinyl groups of Protoporphyrin IX into alcohols; however, this compound is only partially soluble in water. Most of clinically approved PSs like *m*-THPC, an extremely powerful PS commercially known as Foscan or Temoporfin,[53] are poorly soluble in water, despite the presence of four hydroxyl groups.[54] Many efforts have been dedicated to solve the water-solubility problem of *m*-THPC and nanoparticles represent a valuable strategy to improve the hydrophilicity of drugs,[55-57] including PSs (**Figure 6**).[58] Polyethylene glycol (PEG) chains are commonly used in the field of nanotechnology to increase the aqueous-dispersibility of insoluble drugs and prevent their aggregation.[59]

These polymers of nanometric length can interact through multiple hydrogen bonds with water molecules. Moreover, PEG chains increase both the biocompatibility and the lifetime of drugs in the plasma. Finally, due to their elevated molecular weight (*i.e.* high size), they can favor a selective accumulation in cancer tissues.

For example, Ris *et al.* studied how conjugation of *m*-THPC to PEG_{5000} chains can modify its biological properties compared with free *m*-THPC.[60] Water-soluble pegylated *m*-THPC was hence prepared by covalently linking PEG_{5000} chains to each of the four hydroxy groups of the PS. The properties of *m*-THPC and pegylated *m*-THPC were compared in nude mice bearing various types of tumors (namely human malignant mesothelioma, squamous cell carcinoma and adenocarcinoma xenografts), and it was observed that both PSs could photocatalyze the necrosis of the three cell lines, but with different degrees of necrotization.[60] It was found that pegylated *m*-THPC exhibited better photosensitizing properties than non-conjugated *m*-THPC, which was attributed to its enhanced targeting properties toward the tested cell lines. Remarkable accumulation in cancer cells was also observed in another study, in which pegylated *m*-THPC was tested *in vivo* on minipigs bearing mesothelioma xenografts; PEG_{5000}-*m*-THPC can selectively eradicate cells with a tumoricidal effect comparable to that of free *m*-THPC.[61]

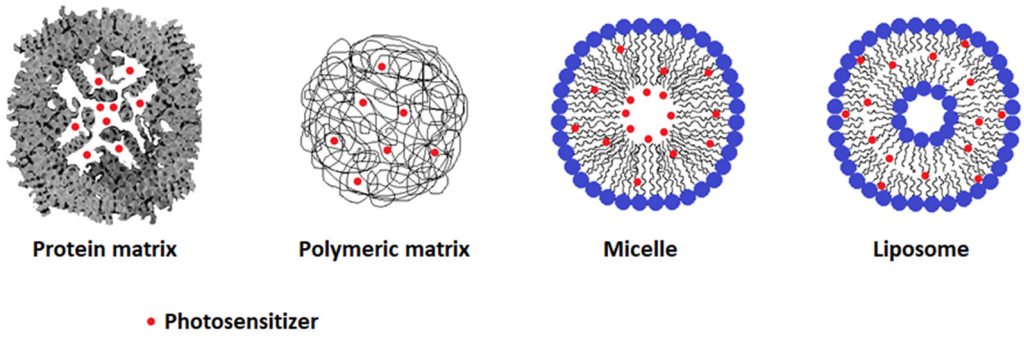

Figure 6. Types of nanoparticles used to improve the water dispersibility of PSs through their encapsulation.

Other studies, instead, have shown that the use of pegylated *m*-THPC is not suitable for treating all types of cancer. For instance, Rovers *et al.* observed that pegylated *m*-THPC evaluated on mice bearing liver tumors exhibited a five-fold decrease in liver uptake compared to *m*-THPC.[62] Pegylated *m*-THPC showed low selectivity since accumulation in normal liver tissues was observed. Although pegylated *m*-THPC displayed liver photonecrosis upon irradiation, the study concluded that they did not present any significant advantages compared to *m*-THPC for the treatment of liver tumors.[62] PEG chains may also be used to improve the aqueous solubility and properties of PSs conjugated to polymers. Thus, Hamblin *et al.* synthesized a polymer conjugated with chlorin e6 (Ce6) and PEG chains, which, in comparison with the non-pegylated polymer, could prevent its self-aggregation and increase the phototoxicity *in vitro* against ovarian cancer cells, *viz.* OVCAR-5 cells.[63] The pegylated polymer was also injected intraperitoneally into nude mice bearing OVCAR-5 tumors, showing a higher accumulation in cancer tissues than the non-pegylated form.[63]

Prevention of the aggregation of PSs and polymer-conjugated PSs may be achieved through encapsulation into nanoparticles. Several studies have been carried out with liposomal nanoparticles, which can contain large amounts of *m*-THPC. A well-known example of such strategy is Foslip®, a liposomal formulation consisting of dipalmitoylphosphatidylcholine liposomes (DPPC) that improve the aqueous-dispersibility of encapsulated *m*-THPC.[64] Moreover, compared to free *m*-THPC, Foslip® presents a better accumulation in tumors,[65] which is a crucial feature as it is recognized that *m*-THPC can damage healthy tissues around the tumors; this is in fact a reason why it was approved by the EMA but not by the FDA

Additional advantages of Foslip® compared to free *m*-THPC are its slightly better cellular uptake by HeLa multicellular spheroids and its non-cytotoxicity in the dark.[66] Foslip® can also be used against tumoral

cells resistant to chemotherapy; for instance, it was tested on 5-fluorouracil-resistant HT29 cells (human colorectal cancer cells), and it was found to induce apoptosis.[67] Improvement of Foslip®, namely Fospeg®, was developed, which consists of a pegylated liposomal formulation with dipalmitoylphosphatidylcholine. As Foslip®, Fospeg® shows selective accumulation in cancer cells, is not cytotoxic in the dark,[65] and does not present significative side effects when administrated to cats.[68] The main advantage of Fospeg® compared to Foslip®, is its longer half-life in plasma as the result of the pegylation of the liposomes' surface. Foslip®, instead, is rapidly degraded in circulation, causing a low bioavailability of *m*-THPC. Another important advantage of Fospeg® compared to free *m*-THPC and Foslip®, is the possibility to modulate the density in PEG chains, which allows to tune the properties of the PS.[69] Furthermore, the properties of Fospeg® may be adjusted through the length of the PEG chains used. For example, Cruje and Chithrani covered the surface of 50 nm-gold nanoparticles with PEG chains of different lengths and densities; it was shown that properties like the cellular uptake and the clearance from the body were dependent on these two parameters (**Figure 7**).[70]

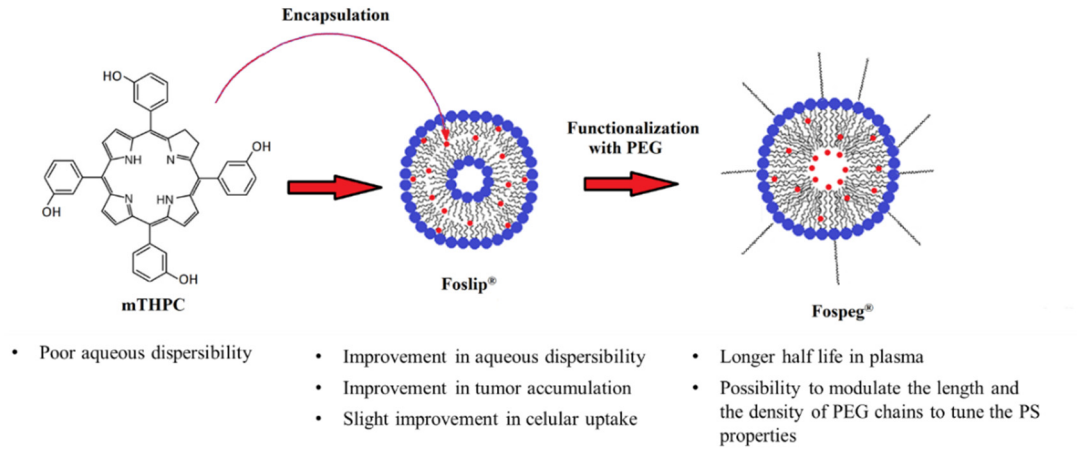

Figure 7. Encapsulation of mTHPC in liposomes (Foslip®) and further functionalization with PEG chains (Fospeg®) guarantees a better performance in comparison with molecular mTHPC.

Extracellular vesicles represent another alternative approach to address water-solubility issues of PSs like *m*-THPC. There are different types of membrane vesicles secreted by living cells; such vesicles have a structure that is like that of liposomes. They are also constituted of phospholipid bilayers but can exhibit enhanced biocompatibility and stability in the blood circulation, by comparison with liposomes. Extracellular vesicles loaded with *m*-THPC have been reported that show better properties than Foslip®, in terms of biodistribution, tumor-cell internalization and photodynamic effect *in vivo*.[71]

Proteins can also be used to increase the water solubility of PSs. For instance, the poorly water-soluble zinc hexadecafluorophthalocyanine ($ZnF_{16}Pc$) was encapsulated in GD4C-modified ferritin (RFRT) and the resulting system was tested with U87MG subcutaneous tumor models on nude mice; good tumor accumulation and tumor inhibition were observed, as well as very low toxicity in normal tissues.[72]

II.1.3. Nanoparticles for light conversion and the treatment of deep-seated tumors

A key factor for the treatment of deep-seated tumors with PDT is the tissue penetration depth of light for the activation of the PSs. Indeed, excited PSs can react with molecular oxygen and consequently initiate a chain reaction producing harmful radicalar species that will kill the cancer cells. The light wavelength used is therefore crucial; although light penetration also depends on the type of tissue irradiated,[73] it is generally accepted that the UV-to-blue-light range shows the lowest ability to penetrate tissues, while NIR has the highest penetration.[74] Many tumors can reach large sizes (≥ 1 cm),[75-78] making the absorption of light at high wavelengths essential, and the NIR window (700–1100 nm) is the best spectral range to

cure large and deep-seated tumors. To date, the number of NIR-absorbing PSs is small and UV/VIS-absorbing PSs are being used to treat superficial or very small tumors. It clearly appears that PDT will only be used extensively when efficient PSs absorbing at high wavelengths will be developed.

A type of NPs that may help to solve this light issue are the so-called upconversion nanoparticles (UCNPs). UCNPs are usually lanthanide or actinide-doped nanocrystal, which can absorb light in the infrared region, and convert it into higher-frequency radiations (shorter wavelengths), in the UV-to-visible region (**Figure 8**).[79]

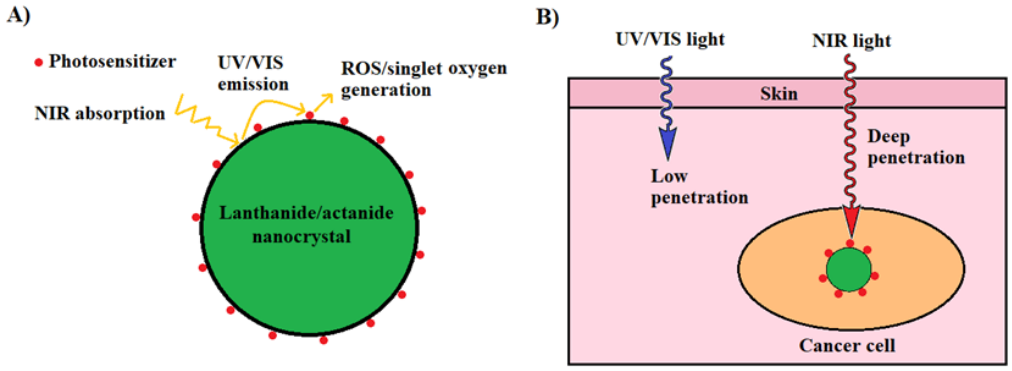

Figure 8. A) Illustration of the upconversion mechanism: the NIR light is absorbed by the lanthanide/actinide nanocrystal and converted into UV/Vis light, which is absorbed by the PS to generate $^1O_2$ or/and ROS; **B)** Schematic representation of the NIR-light penetration in body tissues compared with UV/VIS light.

Thus, conjugation of molecular PSs to upconversion NPs whose emission corresponds to the excitation wavelength of the PSs would allow the treatment of deep-seated tumors through the application of NIR light. However, the field of UCNPs is relatively recent, and the energy-conversion performances achieved until now are still far from optimal. For instance, Boyer and Veggel determined the absolute quantum yields of many UCNPs, and values lower than 3% were found.[80] UCNPs functionalized with PSs have been reported, and despite the low quantum yields, interesting PDT data have been obtained, both *in vitro* and *in vivo*. For example, Gu *et al.* described the preparation of mesoporous silica nanospheres containing CaF_2:Yb, Er nanocrystals entrapped in their porous structure.[81]

The nanoparticles were subsequently coated with a layer of MnO_2, pegylated, and loaded with chlorin e6 (**Figure 9**). These multifunctional nanoparticles could absorb light in the NIR range and convert it into a higher frequency light that can be absorbed by chlorin e6. The MnO_2 layer plays actually a dual role: (i) the Mn^{2+} ions improve the light conversion of the CaF_2: Yb, Er nanocrystals, therefore favoring a better photodynamic effect; (ii) MnO_2 converts endogenous H_2O_2 produced by tumor cells into dioxygen, which is available for the PS under a hypoxic environment. High cellular uptake (with 4T1 cells) was observed with these nanoparticles, which did not show any significant toxicity in the dark compared to free Ce6. Efficient and sufficient phototoxicity were observed with 4T1 cells under normoxic (21% O_2) and hypoxic (1% O_2) conditions, respectively. Finally, they exhibited high a phototherapeutic effect on mice bearing 4T1 tumors upon NIR irradiation, as evidenced by a clear decrease in tumor size.[79]

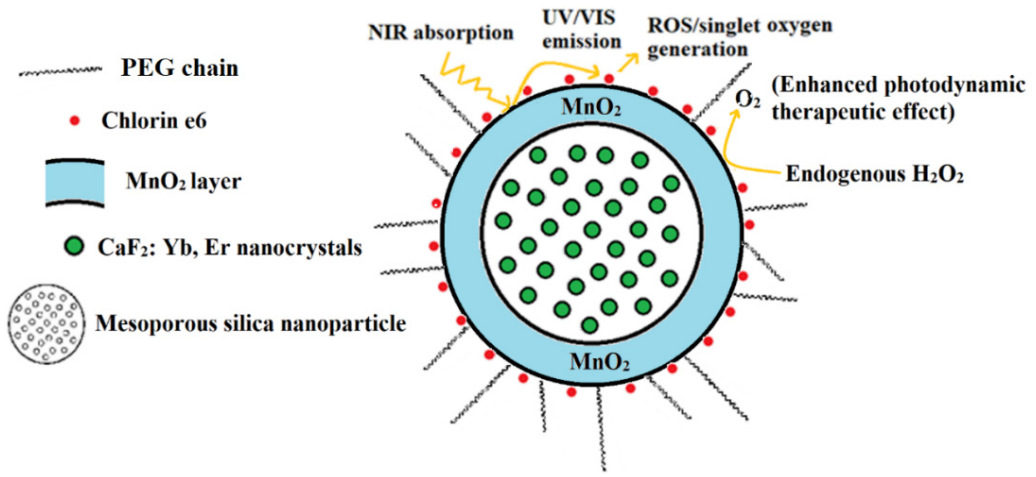

Figure 9. Mesoporous silica nanospheres containing CaF2:Yb,Er nanocrystals and coated with a layer of MnO_2, PEG chains and loaded with chlorin e6.

UCNPs may suffer from low water solubility. To address this issue, Shan *et al.* prepared β-$NaYF_4$:Yb^{3+},Er^{3+} UCNPs and functionalized them with biocompatible poly(ethylene glycol-block-(DL)lactic acid) block copolymers to increase their hydrophilicity and prevent their aggregation. These NPs were subsequently loaded with the PS meso-tetraphenyl porphine.[82] They were tested *in vitro* using HeLa cells (cervical cancer); low dark toxicity was observed and a good ability to kill tumor cells under NIR irradiation was found. Various examples of UCNPs for the potential treatment of the deep-seated tumors have been described in the literature, indicating that this kind of nanoparticles may have a significant impact significantly in cancer phototherapy.[83-85] However, due to the elevated cost of rare-earth elements, cheaper alternatives are examined. For example, nanoparticles that cannot upconvert light themselves, but are conjugated to molecules capable of converting NIR into shorter wavelengths are investigated. For instance, Cheng *et al.* have anchored a fluorescent molecule that can be excited through two-photon absorption, *viz.* fluorescein isothiocyanate, and the PS Pd-meso-tetra(4-carboxyphenyl) porphyrin to mesoporous silica nanoparticles (**Figure 10**).[86] Such co-anchoring inside a well-ordered mesoporous structure allowed to achieve an efficient Förster resonance energy transfer (FRET) between the luminescent dye and the PS, permitting its excitation leading to the conversion of triplet oxygen into singlet oxygen; this system was efficient to kill tumor cells *in vitro* and *in vivo*.[86]

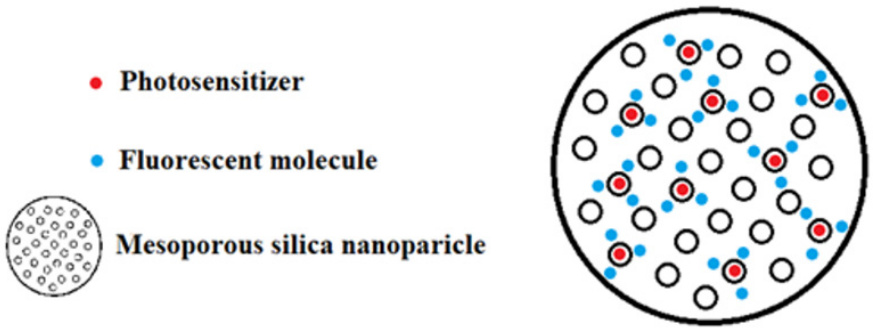

Figure 10. Mesoporous silica nanoparticle in which fluorescent molecules were used as an alternative to lanthanide/actinide nanocrystals to promote NIR upconversion into UV/Vis light.

Recently, polymeric nanoassemblies consisting only of PS are gaining interest because of their high and precise PS loading ratios and negligible toxicity. Chao and coworkers reported glutathione (GSH)-sensitive polymer nanoparticles made either of ruthenium[87] or iridium[88] complexes linked by disulfide bonds for two-photon PDT application. They exhibited enhanced uptake compared to their monomeric

constituents and GSH-dependent release of the PS. Because of the subsequent depletion of ROS-scavenging GSH inside cells, these nanoassemblies achieved excellent two-photon PDT efficiency in vitro and *in vivo*.

Other nanomaterials that are promising to treat deep-seated tumors are quantum dots (QDs) and carbon dots (C-dots). QDs are inorganic semiconductors of nanometric size that have gained much interest for their properties; they typically show intense light emission and high photostability; moreover, their emission peak and absorption wavelength are modulable by controlling their size. They can overcome the limit of low-tissue penetrability since they can absorb NIR light and convert it into visible light through Förster resonance energy transfer (FRET), promoting the excitation of the PSs that are anchored onto their surface. For example, Feng *et al.* conjugated the PS 5-aminolevulinic acid (ALA) to different $CuInS_2/ZnS$ QDs, which were able to promote the indirect excitation of the molecular PSs with a FRET efficiency of up to 58.5% under NIR irradiation. The photodynamic effect of ALA-QDs conjugates were investigated *in vitro* on MCF-7 cells and it was showm that the QD conjugates eradicated most of the cells under 800 nm and 1300 nm laser irradiations.[89] C-dots are another class of nanomaterials with analogous photophysical properties, but in contrast to QDs, they are no afflicted by the risk of releasing heavy-metal ions in biological systems; for this reason, they are considered much safer for biomedicinal applications. For instance, Huang *et al.* described the use of C-dots for simultaneous NIR fluorescence imaging and PDT. They prepared C-dots covered with PEG chains functionalized with chlorin e6 (C-dots-Ce6). Like the QDs described above, they allowed the indirect excitation of the PS by FRET from the C-dots to the PSs. The C-dots-Ce6 were tested on nude mice with subcutaneous MGC803 gastric cancer xenograft and resulted to be excellent imaging agents and exploitable for PDT, since partial tumor regression was observed (**Figure 11**).[46]

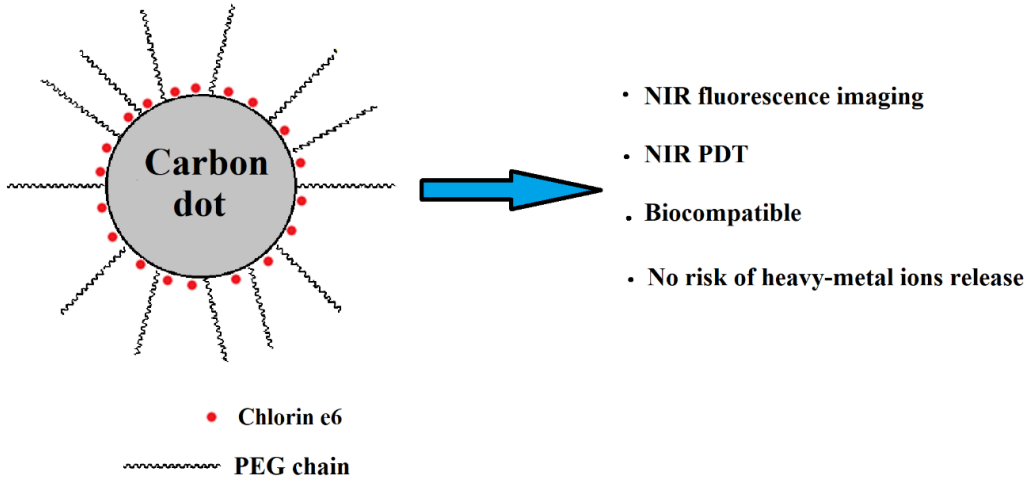

Figure 11. C-dots functionalized with PEGs and chlorin e6 for simultaneous NIR fluorescence imaging and PDT.

A different strategy that is gaining interest to cope with poor UV/Vis light penetration consists in the combination of molecular or nanoparticle-based PSs with X-rays (XPDT). Molecular PSs used in PDT may also be applied in XPDT; for instance, hematoporphyrin derivatives, Photofrin®, protoporphyrin IX, acridine orange, Radachlorin®, methylene blue and metalloporphyrins, have shown good results in preclinical and clinical XPDT studies.[90,91] NPs for XPDT are divided into two types, namely the nanoscintillators and semiconductors. Nanoscintillators absorb X-ray photons and convert them into UV/Vis light that is required to activate the PS. In the case of semiconductors, the X-ray irradiation excites the NP's electrons, and the activated material reacts with the surrounding biological microenvironment, generating $^1O_2$ and ROS. The main advantage of XPDT lies in its ability to penetrate physiological tissues without any limit. Though, XPDT presents lower selectivity compared to PDT; the potential of this technique is restricted by the ionizing nature of X-rays, which can seriously damage healthy tissues close to the treated tumor area.[90]

II.1.4. Nanoparticles for the treatment of hypoxic tumors

PDT proceeds through two main mechanisms, namely the type I and type II mechanisms, type II being the most prevalent one.[92-94] PSs acting through a type II mechanism are more dependent on the presence of dioxygen; therefore, they will logically be less efficient under hypoxic conditions. As a matter of fact, many solid tumors are hypoxic, which is a strong limitation for most PSs. PSs with a type I mechanism can still be effective in such tumors as they can produce harmful species under a dioxygen-poor environment. After PS excitation, type I mechanism starts with an electron transfer from the excited triplet state of the PS to triplet dioxygen, leading the formation of a superoxide anion radical. $O_2^{\bullet-}$ is then converted into dihydrogen peroxide by superoxide dismutase and this H_2O_2 is converted into O_2 by catalase, the two enzymes being present in significant amounts in cancer cells.[95] In this way, molecular oxygen is regenerated and can start a new cycle. Unfortunately, there is currently no well-established method(s) to prepare PSs of the type I mechanism; actually, examples of PSs that function under low O_2 concentrations are scarce in the literature. A possible answer to hypoxia would be the use of nanomaterials able to release or produce dioxygen directly within the tumor environment, so that PSs of type II can then operate.[96,97] As nicely reviewed by Wan *et al.*,[96] five approaches can be considered, namely 1) the delivery of exogenous O_2 directly to the tumor, 2) the *in situ* generation of O_2 in the tumor, 3) the reduction of O_2 consumption by tumor cells through inhibition of the respiration, 4) the regulation of the tumor microenvironment by normalizing tumor vasculature or disrupting the tumor ECM, and 5) the inhibition of HIF-1 signaling pathway to relieve tumoral hypoxia. The five possible strategies have been employed and some interesting *in vitro* and *in vivo* data have been obtained. For example, Hong *et al.* described the tumor oxygenation using a combination of sonodynamic and photodynamic therapy.[98] A nanoemulsion using Pluronic (*i.e.*, a non-ionic surfactant) and perfluoropolyether (PFPE) was synthesized *via* phase inversion composition method, and a PS, namely chlorin e6, was encapsulated, giving nanodroplets dispersed in water (Ce6-P/W NE).[99] Perfluorocarbons present very high oxygen loading efficiency, and PFPE was used as a dioxygen carrier to increase the phototoxicity of chlorin e6. The nanodroplets with a PFPE core showed about 17 times higher $^1O_2$ production in hypoxic solutions through the application of ultrasounds, and about 3 times higher $^1O_2$ production upon light irradiation than an aqueous emulsion of free chlorin e6.[99] The Ce6-P/W NE nanosystem exhibited *in vitro* cytotoxicity under both normoxic and hypoxic conditions, while Ce6 E was only active under normoxic conditions. Hong *et al.* recently developed a multifunctional H_2O_2-responsive and O_2-carrying nanoemulsion with the objective to combine the delivery of exogenous O_2 with *in situ* O_2 generation (**Figure 12**).[100]

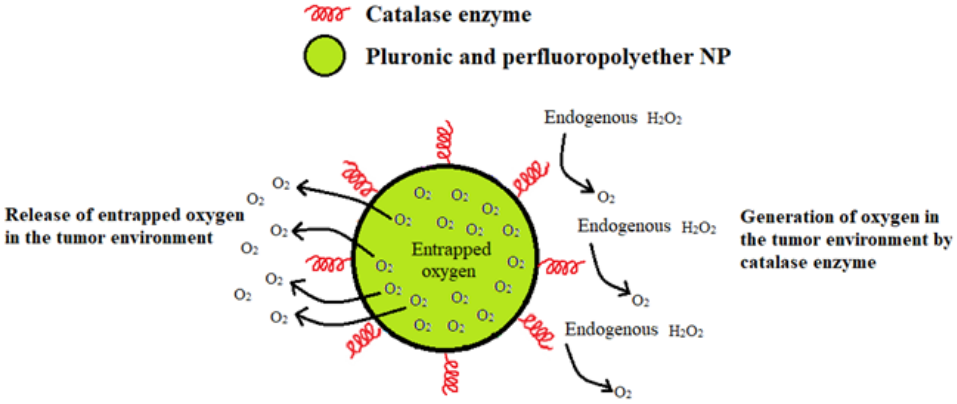

Figure 12. Illustration of a nanoparticle designed to counteract PDT limitation due to hypoxia. This NP acts through the 1) delivery of exogenous O_2 entrapped in the nanoparticle core; 2) *in situ* O_2 generation by decomposition of endogenous hydrogen peroxide produced naturally by tumor cells.[100]

Nanoplatforms, termed as CIPN, were prepared from Pluronic and perfluoropolyether, and the PS IR780 was encapsulated. The surface of the nanodroplets was then decorated with catalase. The activity of CIPN was evaluated and compared with that of IPN, a nanodroplet prepared in the same way as CIPN but without catalase, to assess the ability of CIPN to decompose endogenic H_2O_2 in tumors. The results obtained showed that IPN was not able to produce dioxygen in a solution of H_2O_2, in contrast to CIPN

(*viz.*, through catalase activity). Hence, CIPN showed higher $^1O_2$ generation than IPN when excited with NIR light, and it was able to kill cancer cells (OVCAR-3) with a decrease of their viability of about 39 %.

Apart from the strategies mentioned above, a very elegant new approach consists in using molecules in their endoperoxide forms and directly generate singlet oxygen *via* photothermal stimulation; this strategy does not require the presence of ground-state molecular oxygen in the tumor. For example, Huang *et al.* synthesized a polymeric carrier functionalized with three key elements, namely PEG chains, aza-BODIPY and the endoperoxide form of 1,4-dimethylnaphthalene. Aza-BODIPY acts as a photothermal agent, which can produce heat that mediates the release of singlet oxygen from the endoperoxide form of 1,4-dimethylnaphthalene (**Figure 13**).[101]

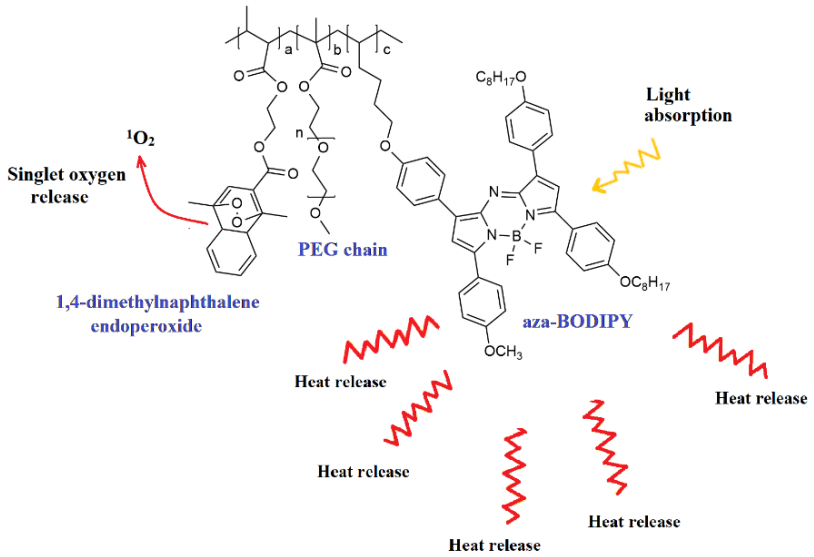

Figure 13. Polymeric carrier functionalized with PEG chains, aza-BODIPY and the endoperoxide form of 1,4-dimethylnaphthalene for PDT and PTT applications.

The multifunctional nanomaterial was capable of efficiently eradicating HeLa cells, especially under hypoxic conditions. Moreover, good inhibitory effect on tumor growth was observed *in vivo* with HeLa tumor-bearing mice.[101] Similarly, Han *et al.* designed an oxygen-independent triblock co-polymer combining photothermal and photodynamic properties.[102] The photothermal agent, namely cypate, and the endoperoxide form of diphenylanthracene were encapsulated in the triblock co-polymer to obtain a system for which the hyperthermia generated by cypate allowed the generation of singlet oxygen from diphenylanthracene endoperoxide. The "smart material" was able to kill cancer cells *in vitro* and showed remarkable antitumoral properties, as evidenced by significant tumor-growth reduction in mice upon NIR irradiation.[102] A comparable system was reported by Li *et al.* who developed Bi_2Se_3 nanoparticles capable of inducing hyperthermia upon NIR irradiation (λ = 808 nm); the heat generated promoted the release of free radicals by decomposition of 2,2-azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride (AIPH).[103] In the presence of the radical precursor AIPH, a more efficient system was obtained; under both normoxic and hypoxic conditions, the AIPH-loaded Bi_2Se_3 (encapsulated or conjugated) NPs showed excellent cytotoxic properties *in vitro* and a tumor inhibition growth of 99.6% was observed *in vivo* (**Figure 14**).[103]

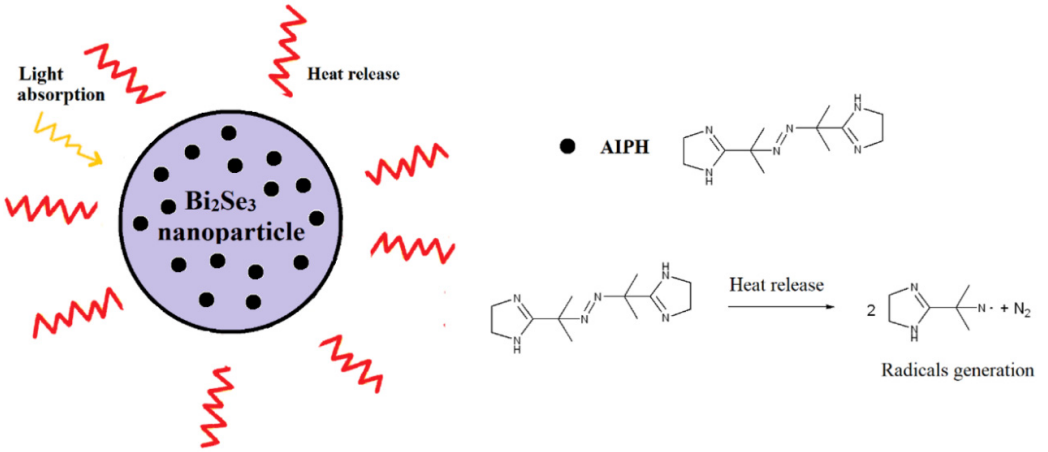

Figure 14. Bi_2Se_3 nanoparticles conjugated with AIPH for the treatment of hypoxic tumors.

II.1.5. Summary

Representative/illustrative examples (mentioned in this minireview) of nano-based approaches to solve the issues encountered with PSs, namely the selectivity and cellular uptake, hydrophilicity, light activation, and hypoxia, are summarized in **Table 1**.

Table 1. Examples of nanosystems that have been developed to improve the properties and therefore the clinical applicability of PDT photosensitizers.

Category	Nanosystem	Coating (additional unit)	PS	Type of targeting (targeting unit)	Mode of treatment (O_2 source)	Refs
NPs to improve targeting and cellular uptake	Polymeric nanoparticles of polylactic acid	-	Hypericin	Passive	PDT	[24]
	Gold nanoparticles	PEG	Zinc–phthalocyanine derivative	Active (Anti-HER2 monoclonal antibody)	PDT	[38]
	PEG-liposomes	PEG	Aluminium–phthalocyanine tetrasulfonate	Active (transferrin)	PDT	[39]
	Silk fibroin nanoparticles loaded with 5-fluorouracil	-	Chlorin e6	Active (cRGDyk peptide)	PDT/Chemotherapy	[40]
	Polydopamine nanoparticles	-	Chlorin e6	Active (Hyaluronic acid)	PDT/PTT	[43]
	$(Zn_{0.4}Mn_{0.6})Fe_2O_4$ nanoparticles	(^{89}Zr for radiolabeling)	Tetrakis(4-carboxyphenyl)porphyrin	Magnetic	PDT	[45]
	Iron oxides nanoparticles	-	Chlorin e6	Magnetic	PDT	[46]
	HPMA copolymer-Mce6 and HPMA copolymer-ADR conjugates	-	Mesochlorin e6	Passive	PDT/Chemotherapy	[104]
NPs to increase water solubility	PEG-*m*-THPC	-	*m*-THPC	-	PDT	[60]
	PEG-*m*-THPC	-	*m*-THPC	-	PDT	[61]
	PEG-*m*-THPC	-	*m*-THPC	-	PDT	[62]
	Chlorin-e6- and PEG-conjugated polymer	-	Chlorin e6	-	PDT	[63]
	DPPC liposomes (Foslip®)	PEG	*m*-THPC	-	PDT	[66,67]
	Pegylated DPPC liposomes (Fospeg®)	PEG	*m*-THPC	-	PDT	[65,68]
	Extracellular vesicles	-	*m*-THPC	-	PDT	[71]
	GD4C-modified ferritin	-	Zinc hexadecafluorophthalocyanine	-	PDT	[72]

NPs for light conversion and the treatment of deep-seated tumors	Mesoporous silica nanospheres	MnO_2 (CaF_2:Yb,Er)	Chlorin e6	-	PDT	[81]
	Metal complex polymeric nanoparticles	-	Ru(II) or Ir(III) complexes	Hyaluronic acid	PDT	[87,88]
	β-$NaYF_4$:Yb^{3+},Er^{3+} nanocrystal	(PEG-(DL)-lactic acid) block copolymers (β-$NaYF_4$:Yb^{3+},Er^{3+})	Meso-tetraphenyl porphine	-	PDT	[82]
	Mesoporous silica nanoparticles	(Fluorescein isothiocyanate)	Pd-meso-tetra(4-carboxyphenyl) porphyrin	-	PDT	[86]
NPs for hypoxic conditions	Nanoemulsion between Pluronic and perfluoropolyether	-	Chlorin e6	-	PDT/SDT (Delivery of exogenous O_2)	[97]
	Nanoemulsion between Pluronic and perfluoropolyether	(Catalase)	IR780	-	PDT (Delivery of exogenous O_2 / *in situ* generation of O_2	[99]
	Polymer carrier conjugated with PEG, aza-BODIPY and DMN	-	-	-	PDT/PTT (*in situ* thermal generation of $^1O_2$)	[100]
	Triblock co-polymer of PEG-bPCL-b-PPEMA conjugated with cypate and DPAE	-	-	-	PDT/PTT (*in situ* thermal generation of $^1O_2$)	[101]
	Bi_2Se_3 nanoparticles conjugated with AIPH	-	-	-	PDT/PTT (*in situ* thermal generation of ROS)	[102]

III. Concluding Remarks

Many PSs have been developed for potential anticancer applications, but their intrinsic limitations impede their wide utilization in PDT as an alternative to chemotherapy or surgery. Nanotechnology may help to address the important drawbacks of traditional PSs and thus promote their increased clinical use. For instance, Visudyne® represents a successful example of a liposome-based nanosystem which encapsulates a potent PS, namely Verteporfin.[105] This encapsulation of the PS solves the aqueous-solubility problem, preventing its self-aggregation and therefore allowing its use in clinics.[106] To date, Visudyne® is the most important milestone of nanotechnology in the field of PDT, since it is the sole clinically approved nanoparticle-based PS used for the treatment of the wet form of age-related macular degeneration (**Figure 15**). Visudyne® also showed great potential as anticancer agent; it completed phase I/IIa for the treatment of primary breast cancer (NCT02872064), phase I for the treatment of vertebral metastases (NCT02464761), and phase I/II for the treatment of patients with melanoma at stage III or IV (NCT00007969). Additional studies have been carried out for the treatment of human retinoblastoma (NCT04429139), pancreatic tumors (phase II; NCT03033225) and recurrent high-grade EGFR-mutated glioblastoma (phase I/II; NCT04590664).

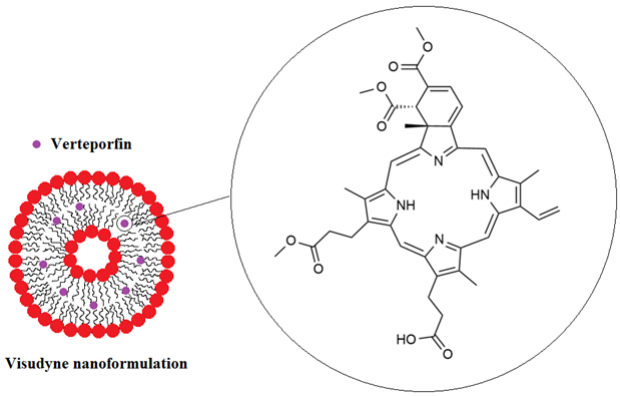

Figure 15. Visudyne nanoformulation for the treatment of the wet form of age-related macular degeneration, in which Verteporfin is encapsulated in liposomes.[105]

There are currently several nanoparticle-based PSs undergoing clinical trials and pre-clinical trials, illustrating the great potential of nanotechnological approaches for the future development of PDT treatments of cancer.[58,107,108]

IV. Conflict of Interests

The authors declare no conflict of interests.

V. Acknowledgements

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