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## Nanophotosensitizers for cancer therapy: a promising technology?

Ancély Ferreira dos Santos , Gabriel S Arini, Daria Raquel Queiroz de Almeida and Leticia Labriola\*

Biochemistry Department, Chemistry Institute, University of São Paulo, São Paulo, Brazil

\* Author to whom any correspondence should be addressed.

E-mail: [labriola@iq.usp.br](mailto:labriola@iq.usp.br)

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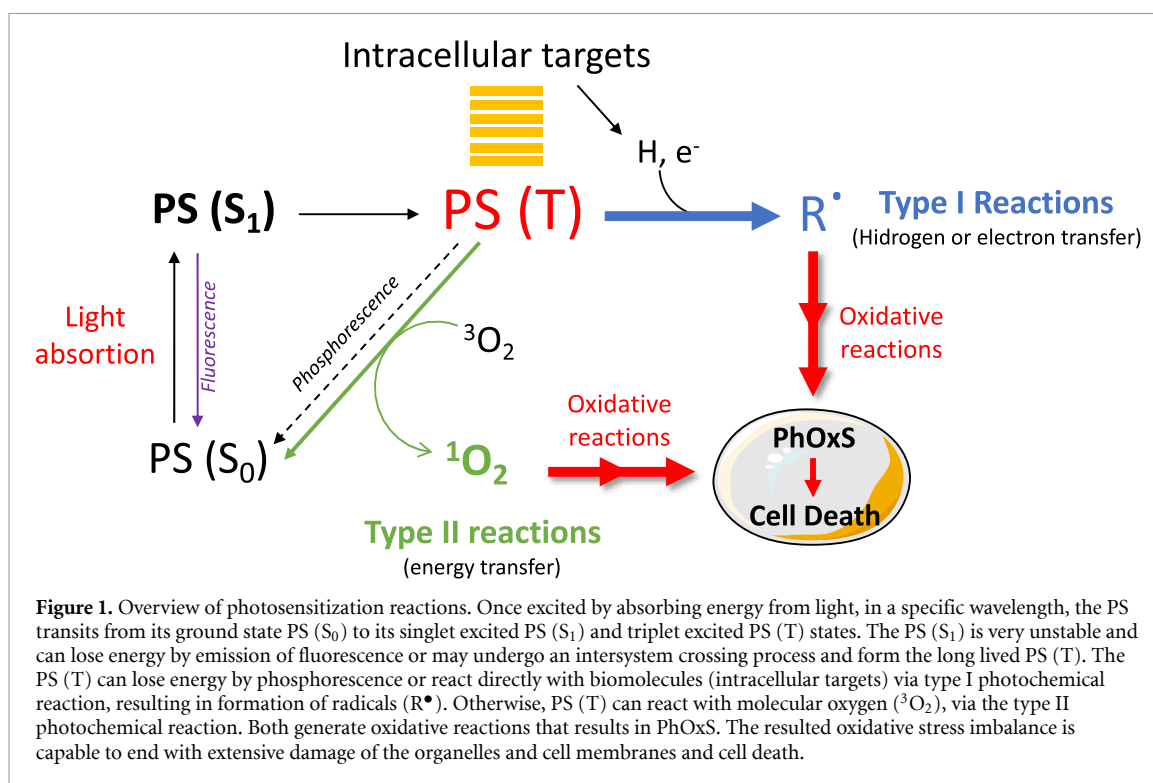
## Abstract

Photodynamic therapy (PDT) has been clinically applied to cure various diseases including cancer. Indeed, photophrin (porfimer sodium, Axcan Pharma, Montreal, Canada), a heterogeneous mixture of porphyrins, was the first photosensitizer (PS) approved for the treatment of human bladder cancer in 1993 in Canada. Over the past 10 years the use of PDT in the treatment of benign and malignant lesions has increased dramatically. However, PDT is still considered as an adjuvant strategy due to its limitations, primarily including low tissue penetration by light and inaccurate lesion selectivity by the PSs. To overcome this scenario, new technologies and approaches including nanotechnology have been incorporated into the concept of PS formulations as PS delivery systems, as PSs per se or as energy transducers. The ideal nanophotosensitizer (NPS) for cancer therapy should possess the following characteristics: biocompatibility and biodegradability without toxicity, stability in physiological conditions, tumor specific targeting, strong near infrared absorption for efficient and sufficient light absorbance and large singlet oxygen quantum yield for PDT. To fulfill these requirements, several nanoscale delivery platforms and materials have been developed. In this review we will focus on the state of the art of nanotechnology contributions to the optimization of PDT as a therapeutic alternative to fight against cancer. For this purpose we will start from the basic concepts of PDT, discuss the versatility in terms of NPS formulations and how to tackle the deficiencies of the current therapy. We also give our critical view and suggest recommendations for improving future research on this area.

## 1. Introduction

Photodynamic therapy (PDT) is an emerging modality for the treatment of a variety of diseases that require the elimination of pathological cells [1–3]. It is based in the uptake of a photosensitizer (PS) molecule which, upon excitation by light in a determined wavelength, reacts with oxygen and generates oxidant species (radicals, singlet oxygen, triplet species) in target tissues, leading to photooxidative stress (PhOxS) [4–6], which results in photodamage of membranes and organelles [7, 8] finally leading to cell death [6, 9, 10].

Like all the newly proposed treatments, there is still place for improvements and lots of resources have been invested in this field [11]. In order to provide efficiency and safety using PDT, it is crucial to deliver the PS in therapeutic concentrations to the target cells (such as tumor cells) while simultaneously being absorbed in only small quantities by nontarget cells, minimizing undesirable side effects in healthy tissues [6, 12]. Blue light can be efficiently used for superficial tumors or infections [13, 14]. However, for an effective light absorption within tissues, the spectra of endogenous PSs like hemoglobin, melanin and water need to be taken into account in order to determine the optical treatment window for PDT (the absorption of light by tissue chromophores limits the wavelength range suitable for PDT to about 650–1200 nm) [15]. To achieve deep penetration into tissue, dyes absorbing wavelengths higher than 650 nm are optimal [4, 15], but one should be aware that PS triplet-state molecules excited by wavelength higher than 850 nm have not enough energy to produce singlet oxygen efficiently. For this reason, approximately 850 nm determine the upper limit for PDT irradiation [15]. In order to maximize the use of PDT for deep tumors, a suitable PS should have a high extinction coefficient in the far-red or near-infrared (NIR) spectral region and a high yield of the



long-lived triplet electronic state (formed from the excited singlet state by intersystem crossing) [12]. However, all these characteristics are not always displayed by the currently available PS [16]. The main obstacle being that most PS have extended  $\pi$ -conjugation systems, making the molecules highly planar and tending to be highly hydrophobic, therefore forming aggregates in an aqueous environment [17]. This aggregation tendency lowers PS photoactivation and consequently the efficiency of PDT. Furthermore, PSs have generally not been found to bind or migrate preferentially to tumor cells, making it difficult to target only the diseased tissue when applying PDT [17, 18]. Considerable efforts have therefore been directed to the design of delivery systems that can improve PS activation specifically at the target cells. It is worth of note that many of them are based in the use of nanotechnology. Indeed, the development of nanoparticles (NP)-based PS (NPS) delivery systems present advantages over conventional PS alone such as improved loading capability and PS concentration at the desired site, higher water solubility and biocompatibility, increased photochemical properties and conjugation with multifunctional modifications capable of enabling both diagnostic and therapeutic functions (theranostic effect) [19, 20].

In this review, we set out to perform a broad up-date on nanotechnology applied to PS for PDT and its current implication in cancer research and treatment. We have covered topics going from the photochemical mechanisms to the NPS formulations and the possibility of taking advantage of the different tumor markers to increasing selectivity and rate of success of the therapy. Moreover we discussed the capacity of these nanoparticles of allowing combined treatments with chemotherapeutic, radiotherapy or immunomodulatory agents.

## 2. Photochemical principles

As depicted in the summarized scheme of figure 1, in dark conditions, PSs are singlet in their ground state energy level ( $S_0$ ) because they have two electrons with opposite spin. After absorbing photons of light, they change to the excited singlet state ( $S_1$ ), one of the electron is promoted to a higher energy level but maintains its spin orientation [16]. Because this excited state is unstable and short-lived (nanosecond), one way that PSs used to return to their ground state is by emitting fluorescence. The latter confers to the PSs their inherent property of acting both as theranostic agents. Another one can be by undergoing an 'intersystem crossing' or spin-flip of the homo electron to produce the long-lived triplet state (microsecond to millisecond range) [15, 21]. The PS triplet life-time (PS (T)) is long enough to allow photochemical reactions with ambient oxygen to occur. These reactions can be divided into two distinct types, type I and type II, photochemical processes [4, 11, 15]. In type I reactions, light energy is transferred from PS (T) to biomolecules in direct-contact through electron and/or hydrogen transfer (radical mechanism) [8, 11]. These processes initiate radical chain reactions, yielding to the formation of superoxide radical anion ( $O_2^{\bullet-}$ )

[16] and aldehyde radicals [8]. The superoxide radical anion could follow two routes: (a) dismutates and produces  $\text{H}_2\text{O}_2$ , which in turn could be reduced, generating hydroxyl radicals ( $\text{HO}^\bullet$ ), via the iron-catalyzed Fenton process, or (b) may react with nitric oxide ( $\text{NO}^\bullet$ ) producing peroxynitrite ( $\text{ONOO}^-$ ), a powerful oxidant belonging to reactive nitrogen species family [22].

This altered redox state leads to an imbalance in cell homeostasis and subsequent damage to biomolecules [11, 17, 23]. Type II mechanism involves the transference of the excitation energy directly from the excited PS to molecular oxygen ( $^3\text{O}_2$ ), resulting in the formation of singlet oxygen ( $^1\text{O}_2$ ), another powerful oxidant. Both types of photochemical reactions generate highly reactive oxidants with short diffusion distance ( $<100$  nm) leading initially to local damage [24]. Direct contact reactions of the PS (T) with biomolecules usually cause severe damage in biomolecules but also can cause photodegradation of the PS [8]. The contribution of each processes depends on the chemical structure of the PS (particularly the redox potential) and on the concentration of ambient oxygen [15, 24]. Type I and type II reactions can occur at the same time and are initiated by the excited PS (T). Moreover, radiative relaxation processes of photoexcited PS can occur and give rise to emission of light by phosphorescence (T to  $\text{S}_0$ ). In all cases, the ability to form triplet excited species is the key step in terms of PS's performance. In summary, the oxidant species generated by PhOxS tend to react with biomolecules in their immediate vicinity and initiate subsequent oxidative chain reactions [6], which in turn result in damage to membranes, proteins and DNA, finally promoting cell death (figure 1).

From all the above explanations, one can easily understand that the outcome of PDT critically depends on the intrinsic photochemical characteristics of the PS [25]. As an example, its hydrophobic nature can promote PS aggregation in aqueous media and therefore reduce light absorption capacity and photoactivity [26]. For this reason, several PSs used in PDT may require carrier systems, such as nanoparticles, to enhance cellular targeting and uptake by the cell [27]. Furthermore, studies have demonstrated that the incorporation of certain nano-scale materials in PS formulations could promote a long-lived triplet-excitation emission under ambient conditions. The new excited triplet state lifetimes (millisecond to a second range) are typically greater than that of conventional PS alone (microsecond to millisecond range) [21]. Therefore, the use of nanotechnology into PS formulations emerges as an attractive tool to either increase tumor-specific NPS uptake and promote ultralong-lived triplet state of PS and can greatly improve photochemical efficiency, PS stability, tumor selectivity and the success rate of PDT [28].

### 3. Technologies behind the NPSs for cancer therapy

Nanoparticles are defined as particles that exist on a nanometer scale, that have at least one dimension below 100 nm [29]. Currently the most common materials used for nano-delivery systems include a variety of polymeric- and metal-carriers, micelles, dendrimers, liposomes or nanotubes [28]. PSs can be loaded into nanoparticles at the same time that the particles are being synthesized via covalent binding, encapsulation or after the particle has been formed [30]. Moreover, PSs can also be encapsulated into the core or the shell of nanoparticles through electrostatic, hydrophobic or by hydrogen bridge interactions [31]. In order to increase the effective uptake of the NPS by tumor cells, as well as their accumulation in the intracellular location of interest, several nano-pharmaceutical formulations have been developed [20, 32]. In general, there are two tumor targeted drug delivery strategies: the active and the passive targeting. In both cases, the physicochemical properties together with the possibility of NPS' surface functionalization as well as the pathophysiological characteristics of the tumor microenvironment (TME) are the parameters used to design drug targeting approaches [33]. The passive targeting routes are more common. They exploit two distinctive characteristics of the TME, namely, the enhanced permeability and retention (EPR) effect and the tumor site acidic conditions [20]. The EPR effect is generally attributed to the rapid growth of cancer cells, which consume local nutrients at a high rate and induce the dysregulated generation of imperfect blood vessels. Leaky pores in these new blood vessels can enhance the penetration of circulating nanoparticles into the tumor environment, whereas the penetration in non-malignant tissues is restricted by the intact vasculature barrier [34]. The acidification of the TME is mostly due to an increase in the rate of glycolysis rate and the subsequent lactate formation. The release of this metabolite is accompanied by the co-transport of protons which are the real responsible for the observed decrease in pH in the vicinity of tumors [35].

The active targeting relies on the use of high-affinity ligands that bind to specific surface molecules overexpressed in tumors' surface [34]. Various ligands have been explored including: peptides (e.g. arginine-glycine-aspartate peptide and epidermal growth factor) [36, 37], proteins (e.g. transferrin and antibodies) [38, 39], aptamers [40], vitamins (such as folic acid and biotin) [41] and carbohydrates [42]. The specific ligand-receptor interactions will then promote their internalization via specific cellular pathways [31]. Moreover, the greater advantage of such surface modifications is the reduction of NPS clearance from the liver and spleen [43].

### 3.1. Targeting tumor cells with organic based nanoformulations

Organic nanoparticles present the advantages of greater biocompatibility and biodegradability [19] which could be used to optimize drug release while minimizing off-target toxicity [44]. Common organic nanoformulations use liposomes or polymeric micelles, based on the self-assembly of components that display amphiphilic structures in water with a hydrophobic core, enabling the accommodation of hydrophobic compounds [45]. Liposomes are sphere-shaped vesicles consisting of one or more phospholipid bilayers thereby allowing the encapsulation and transport of both hydrophilic and hydrophobic PS [19, 46]. Phospholipids or polymers, including FDA-approved synthetic ones such as polylactic acid, polyglycolic acid and polylactic-co-glycolic acid (PLGA), are popular materials for the assembly of NPS [47] which may be employed alone or in combination with other compounds. Among them one could cite the well-known agent doxorubicin and selenium. The combination of selenium with organic materials allows the variation of physiochemical and biological properties leading to improved drug loading capacity and permeability of the particles [48]. Indeed, selenium nanoparticles were reported to present less hepato/renal toxicity in mice submitted to an oral dose of 2 mg Se/kg body weight/28 d than animals treated with either inorganic or organic selenium [49]. A recent study performed by Xie *et al* showed enhanced pharmacokinetics and anticancer effect of doxorubicin by using a drug delivery system based in selenium-functionalized liposomes [50].

Potent hydrophobic PSs such as benzoporphyrin derivative (BDP) and zinc (II) phthalocyanine and meta-tetra (hydroxyphenyl) chlorin (m-THPC) benefits greatly from nanolipid formulation in liposomes which allow them to improve their water solubility, pharmacokinetic profile and *in vivo* PDT efficacy [51]. Among the nanoencapsulated PSs, visudyne is an example of nanolipid-based formulation employing a combination of egg phosphatidylglycerol, dimyristoyl phosphatidylcholine, ascorbyl palmitate and butylated hydroxytoluene which has been passed through clinical trials presenting encouraging results [52]. Another major motivation for encapsulating hydrophobic PSs in liposomes is to retain their fluorescence activity (photoactivity) and improve the delivery of a photoactive PS since they typically tend to stack and form J-aggregates in aqueous environments, thus quenching their fluorescence and limiting their photochemical potential [52]. However, due to rapid disintegration and short plasma half-life, conventional liposomes are less effective in establishing elevated tumor-to-normal tissue ratios [53] independently of tumor cell models or PS types [19]. To overcome this issue, long-circulating liposomes have been obtained by introducing modifications in the surface of the nanoparticles resulting in enhanced plasma stability [53]. The most commonly used ligand in this type of liposomes is polyethylene glycol (PEG). This hydrophilic polymer has been shown to stabilize liposomes by the establishment of a steric barrier which improves the efficacy of encapsulated agents by reducing *in vivo* opsonization with serum components [54]. The combination of phospholipids in PEG-based formulations has also been used to increase tumor targeting. For example, the use of methoxy polyethylene glycol (MePEG) conjugated with the PS chlorin e6 (Ce6) promoted increased Ce6 aqueous solubility with *in vitro* enhanced phototoxicity, higher reactive oxygen species (ROS) generation and efficient delivery to colon cancer cells [55]. This formulation also presented *in vivo* improved tumor tissue penetration and intracellular accumulation. In a recent report, the folate-modified PLGA nanoparticles were formulated to target the surface of many cancer cells which display folate receptors overexpression [56]. In this context the use of PLGA nanoparticles loaded with pheophorbide a, an hydrophobic PS, have shown enhanced PDT effect by presenting an increased loading capacity and thus and enhanced killing effect in both human gastric cancer cells and tumor-bearing mouse model [57].

Hyaluronic acid (HA) is commonly used for PSs' encapsulation. It has been shown that PS-loaded HA-based nanoparticles (HANPs) rapidly release the PS in the tumor tissue due to the degradation of the HA backbone in presence of hyaluronidase, an abundant enzyme localized the cytosol of cancer cells [58]. Studies performed in human glioblastoma, mouse colon carcinoma and human breast tumors have also reported a successful increase of HANPs loading capacity and the corresponding higher concentration of PS being specifically delivered into the tumor tissue [59–61]. Recently, HA-containing nanoparticles has been explored to target CD44 positive cells since this glycoprotein is overexpressed in different types of cancer such as some types of breast tumors [62], hepatocellular and colorectal carcinomas where it is associated with poor prognosis [63]. By using HA-decorated polydopamine nanoparticles conjugated to CD44 receptors and loaded with Ce6, Wang *et al* were able to promote improved tumor selectivity, increased cell death and overall treatment outcome in both 2D cell cultures and xenographs of human colon cancer cells [64].

### 3.2. Targeting tumor cells with inorganic based nanoformulations

Inorganic formulations display quite unique magnetic (e.g. iron oxide) and catalytic properties [65]. They are in general, easily synthesized and can be functionalized with moieties and ligands in order to increase their affinity and selectivity toward receptors or target molecules present in cell membranes [19]. Regarding PDT, they can display unique size-tunable optical properties that can match with the working region of PSs



enhancing their photochemical properties [19, 30]. This is the case of porphyrin derived PSs, which are well characterized and most frequently used PS in cancer treatment. Conjugation of them with polymers or metal ions chelated onto their surfaces can lead to enhanced performance [30].

All inorganic NPs share a typical core/shell structure. The core can contain metals (iron oxide, gold and quantum dots) or organic fluorescent dyes encapsulated in silica. This region defines the fluorescence, optical, magnetic, and electronic properties of the particle. The shell is usually made of metals or organic polymers that protect the core from chemical interactions with the external environment and/or serves as a substrate for conjugation with biomolecules, such as antibodies, proteins and oligonucleotides [66]. Several of these inorganic nanoparticles are sufficiently small (10–100 nm) to easily penetrate the capillaries and be up-taken by distinct tissues. Larger particles need to be delivered at the required anatomic sites where they will undergo passive targeting. A clear advantage of inorganic nanoparticles is the possibility of being photo-activatable without the need of releasing the PS [67]. However, despite that these type of NPs seem very promising, one should be aware that Fe-based nanomaterials might have a deleterious impact on normal cells, especially immune cells [68].

Silica-based nanoparticles are good examples that summarize quite well this advantage. Since their size and shape can be well controlled, they present high stability and are easily functionalized [69]. Different synthetic methods can be used to produce a variety of silica-based NPS such as hollow silica, organically modified silicate, mesoporous silica and other nanoparticles made by sol–gel methods [70]. Mesoporous silica consists of a large number of empty pores where active moieties can be incorporated in large amounts. Nanoparticles of this material offer a more homogenous structure, lower polydispersion, and higher surface area for the chemical or physical adsorption of molecules [30]. Silica-based nanoparticles display not only good water solubility but also a high degree of biocompatibility [67]. PS such as m-THPC [71], protoporphyrin IX [72], and methylene blue (MB) [73] have been encapsulated in silica nanoparticles showing higher concentration at the tumor tissue due to the EPR effect [74]. Additionally, the encapsulation of PS allowed their administration in a monomeric form significantly improving PS photostability without any loss of activity [74]. It is important to note that encapsulation significantly improved MB photostability, while surface conjugation diminished it [73]. A recent study reported a substantial increase in singlet oxygen quantum yields ( $\Phi\Delta$ ) with effective ( $\Phi\Delta_{\text{eff}}$ ) exceeding that of MB alone in two types of ultra small PEG-coated (PEGylated) fluorescent core–shell silica nanoparticles where MB was encapsulated into the matrix of the silica core or grafted onto the silica core surface [73]. Additionally, energy-transferring silica nanoparticles for two-photon (TP)-excited PDT can be applied to the diagnosis and therapy of cancer as well as for other diseases or in ultrasensitive bioimaging under the extremely low scattering of TP irradiation displaying drastically lowered side effects compared with those used in chemotherapy [74].

Gold, silver, and platinum displayed broad optical properties and can be easily functionalized with different chemical moieties [75]. In particular, one can highlight the characteristic plasmonic properties of gold nanoparticles (AuNPs) [76]. Unlike other inorganic metallic nanoparticles, AuNPs can be prepared with different geometries, such as nanospheres, nanoshells, nanorods, or nanocages [77]. Their geometric versatility is one of the reasons why they are widely used in targeted drug and gene therapy strategies. Regarding PDT, they present advantages such as the possibility of PSs' incorporation in multi-component delivery systems, as well as the facilitation of PSs' transcytosis across epithelial and endothelial barriers. Additionally, AuNPs can enhance not only the cellular PSs uptake but also the singlet oxygen generation and thus PDT's efficiency [18]. However, AuNPs display high tendency to aggregate. To overcome this limitation it has been reported that coating the AuNPs with hydrophilic polymers, such as PEG or HA, can not only increase their stability but also decrease the overall biotoxicity [31, 70].

Metalloporphyrins containing zinc (Zn) also provide a higher PDT efficiency when compared with porphyrins alone, due to their metallic photothermal contribution [75]. An interesting report showed that AuNPs loaded with the synthetic metalloporphyrin Zn(II)meso-tetrakis(4-carboxyphenyl) porphyrin were able to generate remarkable amounts of singlet oxygen responsible for the cytotoxic effect observed in breast tumor cells after being submitted to PDT treatment [78].

Quantum dots (QDs) are other promising inorganic materials for PDT field. QDs are nearly spherical fluorescent semiconductor nanoparticles with a diameter of 2–10 nm [79]. Compared with organic dyes including conventional PS, QDs have more intense and broader light absorption, higher photobleaching thresholds and photoluminescence quantum yields, and longer photoluminescence lifetimes [80]. Increase knowledge in the preparation, stabilization, and characterization of these systems has promoted their use in PDT. By tailoring their shape, size, and composition, QDs can be engineered to emit at wavelengths that can span the entire spectrum [19]. Therefore, the conjugation of QDs with PS allows the use of excitation wavelength different than the ones absorbed by the PS alone. In this configuration, QDs can generate ROS via type I and type II pathways, and there are reports of direct formation of superoxide radical anions by electron

transfer from photoactivated cadmium and indium-based QDs [80, 81]. Alternatively, photoactivated QDs can act as energy donors and promote PSs' transition to their excited singlet state by transferring energy via Förster resonance energy transfer mechanism [82, 83]. In this case, the PS molecules attached to the particles or present in the colloidal medium can be activated by radiative or non-radiative energy transfer.

Typical QDs usually consists of a semiconductor core and a shell, which protects the core from oxidation and enhances quantum yield. In general they have a cadmium selenide core covered with a zinc-sulfide (CdSe/ZnS) shell [84]. To improve the solubility and specificity of PS for tumor delivery, the surface of the QDs can be modified with thiol ligands or amphiphilic copolymers, which facilitate the superficial conjugation of antibodies or small molecules [30]. It was already demonstrated that the absorption spectrum of polyanionic phthalocyanines (PCs) in complex with QDs was almost equal to the sum of absorption spectra of the individual substances. However, the interaction of QDs with polycationic PCs can lead to a significant disturbance of the PS's spectrum. For example due to electrostatic interactions CdSe/ZnS QDs form stable complexes with polycationic aluminum PCs in aqueous solutions [85]. In these conditions, a highly efficient nonradiative energy transfer from QD to PC was observed, leading to a sharp increase in the effective absorption cross section of PC. When these hybrid complexes are excited within these bands the intensity of PC fluorescence and the rate of  $^1\text{O}_2$  generation increases significantly compared with free PC at the same concentration [85].

A major drawback with the biomedical use of QDs is that they generally contain restricted heavy metals which severely hinder their clinical translation due to the possible release of toxic ions such as  $\text{Cd}^{2+}$ . Indeed the presence of this ion can induce undesired oxidative stress by the massive generation of ROS if it is present as a free ion intracellularly [86]. To overcome this issue, studies have focused in the development of cadmium-free based QDs nanoparticles demonstrating their safety in rodent models. This new strategies can allow the translation of QDs nanoparticles into clinical use [87]. For example, a photoactivatable surface based on polyurethane, a polymer widely used in medical device applications, embedded with a combination of crystal violet as PS and an indium-based QDs was developed and had its effective antibacterial activity demonstrated [80]. In this study the authors showed that interaction between the QDs and the crystal violet occurs within the polymer and leads to enhanced generation of ROS under visible light photoactivation. Hopefully, these alternative QDs could be soon used for cancer research and treatment.

### 3.3. Functional activatable NPS

The use of specific microenvironment physical and/or chemical conditions and light-controlled activatable materials are being used to combine PDT and photochemical internalization (PCI) of cytotoxic molecules. Nanoparticles that are internalized by endocytosis are found in the acidic environments of endosomes (pH 5.0–6.0) and lysosomes (pH 4.5–5.0) [88]. In such environments the degradation of pH-sensitive nanoparticles is accelerated and the conjugated PS released [51]. With the same idea, some strategies of drug-specific delivery takes advantage of particular features of the TME and/or the tumor itself such as pH [89, 90], hypoxia [35], ATP concentration [91], leaky vasculature [92], overexpression of certain enzymes [93], and ligands for promoting the specific release of the PS and the cytotoxic molecule in the tumorigenic cells further increasing the success rate of each therapy alone [94].

For example, nanoparticles with pH-triggered drug release properties have been reported as suitable platforms for tumor-targeted intracellular chemotherapeutic agents delivery [95]. This can be achieved because interstitial fluid in tumors is known to have a lower pH (pH 6.3–7.0 range) than that of normal tissue (pH 7.2–7.6 range) [96]. Accordingly, a recent report has described the use of functionalized zinc(II) phthalocyanine (ZnPc) conjugated with stellate mesoporous silica nanoparticles (SMSNs) (SMSN-ZnPc) [90]. Since the PS was conjugated to the Zn by an acid-sensitive hydrazone bond, when the nanoparticles reached the more acidic ambient present in tumors, they were specifically dissociated in the intracellular space of cancer cells. The authors have found that NPS in this unconjugated state presented increased fluorescence intensity and singlet oxygen generation when compared with PS alone. This approach has been used in *in vivo* models where it has promoted efficient inhibition of cervical tumor growth [90].

Uthaman *et al* have described the effects of the conjugation of the PS Pheophorbide A to a water-soluble glycol chitosan through a ROS-sensitive thioketal linker. During systemic circulation the NPS formed by the self-assembly displayed aggregation-induced self-quenching effect on photoactivity. However, once they were localized within the intracellular environment, the more reducing milieu of tumors enabled photoactivation of the PS. This switchable photoactivity was responsible for the lower phototoxicity in non-tumorigenic sites resulting in increased tumor specificity [97].

However, one of the drawbacks of this strategy is that small variations in the intracellular tumor milieu can jeopardize the activation mechanisms and thus the success of the therapy [17]. Another limitation of this specific tumor-microenvironment-dependent activation strategy is the lower kinetic of *in vivo* release of the PSs from the nanoparticle. This issue needs to be considered in order to achieve effective treatment outcome

[98]. To overcome this limitation, strategies based on controlled light-activation has been explored. In this sense, the use of nanomaterials have also been proposed as an alternative approach to promote energy transfer-mediated PDT.

Up-conversion nanoparticles (UCNPs) are luminescent materials that transform NIR (lower energy) into visible radiation (higher energy). Compared with organic fluorescent dyes and QDs, UCNPs have their own advantages, including the attractive optical features such as sharp emission lines, long lifetimes ( $\sim$ ms), large anti-Stokes shift, superior photo-stability, high detection sensitivity, non-blinking and non-bleaching. Additionally, the use of NIR light to excite photoluminescence in UCNPs enables deeper penetration in biological issues and allows for noninvasive photo-treatment. Moreover, compared to UV or visible light, NIR has much lower energy, which causes fewer damage to tissue and generates weaker autofluorescence from biological background [99]. UCNPs can be used as transducers to activate a PS that is sensitive to visible light by using a NIR light source. The energy from excited states of UCNPs can be absorbed by the PS localized on their surfaces through the transfer of electronic excitation energy either radiative (i.e. absorption of upconverted luminescence photon by PS) or non-radiative (i.e. via Förster or Dexter mechanisms of electronic excitation energy transfer) [95]. Therefore, UCNPs can tackle the problem of limited penetration depth due to light absorption and scattering of biological tissues, and represent a promising alternative for PDT use in cancer treatment [100]. The above listed advantages of UCNPs can indicate their success in *in vivo* applications. However, there are still several problems that need to be solved to make them acceptable for clinical use. One of this is the fact that the excitation wavelength of several UCNPs comprises a band around 980 nm, which might pose a risk concerning water absorption and therefore tissue overheating effects [101]. Additionally, the small absorption cross sections of UCNPs sensitizers are a bottleneck for obtaining intense upconversion emissions and high power density lasers are needed as the excitation sources. This can also contribute to possible heating effects. To solve this issue, efforts have been recently undertaken to design new effective UCNPs structures capable to improve the upconversion emission efficiency. For example, Lee *et al* [102] have recently showed that is it possible to have a successfully energy transfer between PS and UCNPs and reduced overheating by using an UCNP structure with an active shell including Yb and Nd ions for 808 nm triggered upconversion luminescence, instead of the 980 nm wavelength commonly needed. However, more studies are still necessary to avoid PS leakage and long term instability.

In an attempt to combine PDT and current cancer therapies by using light-activatable nanoplatfroms, Shao *et al* have designed a core-shell consisting of an individual UCNP core and a shell of porphyrinic metal-organic frameworks (MOFs), displaying the hypoxia-activated prodrug tirapazamine (TPZ) encapsulated in the nanopores of the UCNP structure [103]. The heterostructure allowed efficient energy transfer from the UCNP core to the MOF shell in such a way that NIR light was able to trigger the production of cytotoxic ROS that promote the release of TPZ and effective colorectal carcinoma treatment both *in vitro* and *in vivo*. Furthermore, they demonstrated that the integration of this nanoplatfrom with immunotherapy using the antiprogrammed death-ligand 1 ( $\alpha$ -PD-L1) checkpoint-blockade therapy led to the complete growth inhibition of untreated distant tumors by inducing specific tumor infiltration of cytotoxic T cells [103].

Although further studies are still needed to better understand the photophysical mechanisms behind efficient energy conversion for *in vivo* applications, the examples commented above illustrate how nanotechnology can be applied to improve photophysical and photochemical properties of the PS in an attempt of overcoming the present limitations of conventional PDT. Particularly, this alternative aims at providing a more efficient therapeutic approach for the treatment of deep lesions by circumventing the low penetration depth of visible light, by increasing tumor selectivity as well as by inducing an efficient immune response against the tumor [17].

#### 4. NPSs in cancer therapy up-to date studies

Since the PS porfimer sodium (photofrin) was first approved for PDT clinical application in 1995 [104], PDT has been used for the treatment of various cancers worldwide [17]. However, only a few PSs have been successfully explored clinically for cancer treatment [11]. Researchers who are in the field of PDT in cancer treatment still need to tackle three main aspects which can be summarized as: (a) guarantee that the PS will reach specifically the tumor-located site, (b) the low penetration capacity of light in the tissues and in some cases (c) the low redox imbalance generated by some PSs [105]. The use of NPS as delivered systems is a promising approach in PDT because it can overcome the limitations described above. For example, NPS can be design to promote the precise accumulation at the tumor site as well as their capacity to enhance PS cytotoxic effects.

Regarding the NPS used in clinical trials, visudyne was the first one approved photonanomedicine formulation for PDT in 2000 [106]. Unfortunately, it is still the only formulation approved for use in Europe



and United States [52]. Visudyne formulation uses verteporfin, a BDP as PS. Visudyne in an aqueous liposomal formulation is the result of almost two decades of advancements in light activatable nanotechnologies. Although initially approved for PDT to treat macular degeneration, the clinical use of visudyne in oncology is rising. It has presented successful results in combination with liposomal cisplatin delivery in clinical trials for pancreatic, prostate, primary and metastatic breast cancers and in lung cancer (NCT03033225, NCT03067051, NCT02872064, NCT02939274 and NCT02702700) [52].

Since the need of increased control over activity, spatiotemporal drug release and selectivity in the targeted delivery of agents as well as the availability of new formulations that allow multi-agent co-encapsulation are still unmet by conventional nanoformulations, there is a huge offer of novel advances in nanomedicine. In table 1, we have summarized the spectrum of recent NPS research studies performed in a variety of cancer models. One can easily realize that the more prevalent ones are breast cancer, cervical and colorectal carcinomas.

## 5. Recommendations for further research and development

Clinical application of nanotechnology coupled to PDT present the advantage to be used in combination with surgical resection of the tumor, when this is possible, radiotherapy, chemotherapy and immunotherapy as already exemplified in the previous paragraphs. Nevertheless this approach still presents some limitations. From a biological point of view there are drawbacks in most of the NPS new formulations studies impairing the shortening of the development process. We will discuss the challenges and how to improve the future research on this area.

### 5.1. Cells and their phenotypic particularities

The development of novel NPS should take into account not only the clinical requirements but also the particularities of each type of tumor. The functional activatable NPS are a good example of these novel approaches. However, it has been demonstrated that different cells, even from the same tumor type, exhibit a great variability in active metabolism pathways and macromolecular composition in their membranes, being able to respond differently to the same treatment [6, 141, 142]. Therefore an urgent shift in the focus of the development strategy from an only sensitizer-centered approach to a tumor type-centered one is needed.

Chemotherapy, radiation, targeted therapies and even PDT presented major advances in patient management over the past decades but refractory diseases and recurrence remains the major reason of treatment failure [141, 143, 144]. This is partly explained by the ability of tumor cells to adapt to potentially harmful situations such as (a) the inherent tumor heterogeneity and clonal evolution of drug resistant cell populations submitted to chemotherapeutics exposure, (b) the number of phototherapy sessions, (c) the delivery system used, and (d) photo-physical aspects of the PS [145, 146]. However, it was already shown that some cells that are resistant to chemotherapy can be slightly more susceptible to PDT [10]. For example, a highest sensitivity to PDT using MB or hypericin as PS was observed in the high aggressive and invasive breast tumor subtype, the triple negative breast cancer, for which there are no targeted treatments, than in less aggressive subtype of breast tumor [6, 141]. For this reason, aligned knowledge of cancer cell biology and in NPS formulation could positively impact the role of overall PDT on cancer therapeutic outcomes. Another example can be retrieved from the several approaches using PDT, either in combination or not with surgery, being tested in pancreatic tumors that are refractory for conventional chemotherapy treatment [147, 148].

Once cell membranes integrity is necessary for survival, therapeutic strategies that triggers specific oxidative damage in the membranes of cell organelles display great potential to avoid therapeutic resistance [146, 149]. In this scenario a clear observation of the differences in the cellular composition between normal and tumor cells present a great value once it could help of the selection of the best NPS for a given cellular composition enhancing the probability of being incorporate by tumorigenic cells. Recently, it was shown that breast tumor cells displayed different lipid composition [150]. This characteristic was associated with the differential cell type response obtained upon PDT using MB as PS. The authors showed that more aggressive cells, which present a high amount of polyunsaturated fatty acid were prone to enroll lipid peroxidation and so ferroptosis as main cell death mechanism and that was the reason for the increased efficacy of the treatment [6]. With this in mind, it would be interesting to evaluate whether a lipid-specific interacting NPS could be more efficient in targeting hard-to-treat cancer cells. For example, Sang *et al* [108, 112] have recently reported the development of specific nanostructures capable to induce cell death in a lipid peroxidation dependent-manner.

### 5.2. Molecular mechanisms behind photodynamic-induced cytotoxicity

Compensatory signaling also influence the molecular mode of resistance where cancer cells activate alternative pathways to escape treatment and inhibit cell death [11, 151]. Regarding the evaluation of the

**Table 1.** Nanotechnology approaches used for PDT in cancer.

Tumor type	Cell line	PSs (wavelength of excitation)	Nanoparticle material (NP size nm)	Ref.
Bladder	MB49 (mouse)	Indocyanine green (808 nm)	Salmonella typhimurium YB1 (50 nm)	[107]
Breast	4T1 (mouse)	Cy7-hex (880 nm)	SPION (40–80 nm)	[108]
		Rose bengal (808 nm)	PEGylated UCNPs@mSiO <sub>2</sub> (42 nm)	[109]
		Ce6 (660 nm)	Human serum albumin (76 nm)	[91, 110]
		IR780-hex (808 nm)	MnO <sub>2</sub> hybrid hydrogel (110 nm)	[111]
	EMT6 (mouse)	m-THPC (650 nm)	Magnetic iron oxide nanoparticles (50–100 nm)	[112]
			Hydrophilic polyphosphoesters (160 nm)	[113]
			Liposome (111–114 nm)	[114]
	MCF-7 (human)	Triphenylphosphine (980 nm)	TiO <sub>2</sub> -coated UCNP (28 nm)	[115]
		Ganoderic acid A (880 nm)	Gold nanorod-loaded liposome (400 nm)	[116]
	MDA-MB-231 (human)	MB (664 nm)	Zirconium phosphate	[117]
Cervical	HeLa (human)	Genipin (240 nm)	Dopamine (110 nm)	[118]
		Zinc(ii)phthalocyanine (610 nm)	Stellate mesoporous silica (100 nm)	[90]
		Tetraphenylporphyrin (419 nm)	Conjugated polymers and gold nanorods (30 nm)	[119]
		Fluorinated PS (660 nm)	Polymeric-organosilica (90 nm)	[120]
		Apt-HyNP/BHQ2 (400 nm)	Hybrid micellar (46 nm)	[121]
		Organic carboxylic molecules nano-hybrids (808 nm)	Layered double hydroxides (50 nm)	[21]
		Zinc(II)phthalocyanine derivative (670 nm)	Mesoporous silica (50 nm)	[122]
		Pyropheophorbide-a (655 nm)	Polycaprolactone and pluronic F-68 (17 nm)	[123]
Cholangiocarcinoma	HuCC-T1 (human)	Ce6 (664 nm)	Ursodeoxycholic acid-conjugated chitosan (200–400 nm)	[124]
	SNU478 (human)		Water-soluble chitosan (300 nm)	[125]
Colorectal	CT26 (mouse)	Ce6 (664 nm)	MePEG (50–100 nm)	[55]
			Thiodipropionic acid-phenyl boronic acid pinacol ester conjugated with chitosan-g-methoxy PEG copolymer (200 nm)	[126]
			HA (126 nm)	[60]

(Continued.)

Table 1. (Continued.)

Tumor type	Cell line	PSs (wavelength of excitation)	Nanoparticle material (NP size nm)	Ref.
		5,10,15,20-tetra p-benzoato porphyrin (650 nm)	MOFs (100 nm and 150 nm)	[127, 128]
				[128]
		Porphyrinic MOF (980 nm)	UCNP (38–65 nm)	[103]
	HCT-116 (human)	Ce6 (664 nm)	HA (126 nm)	[60]
			Biodegradable copolymers	[89]
			PEG-polyethylenimine conjugate (150 nm)	[129]
Fibrosarcoma	HT1080 (human)	TiO <sub>2</sub>	PEG, coated with transferrin (18 nm)	[38]
Glioma	U87MG (human)	Ce6 (664 nm)	HA (126 nm)	[60]
	CCL-107 (rat)	MB (664 nm)	Polyacrylamide (78.5 nm)	[130]
Hepatocarcinoma	HepG2 (human)	Phthalocyanine (610 nm)	Silicon(IV)-biotin (100 nm)	[131]
		Tetraphenylporphyrin (419 nm)	Polymer (80 nm)	[132]
	Hepa1-6 (mouse)	C60 (980 nm)	Monomalonc modified UCNP (34 nm)	[133]
Lung	A549 (human)	Rose bengal, zinc phthalocyanine	Poly(allylamine) modified UCNPs (8 nm)	[134]
	LLC (mouse)	Zinc phthalocyanine (980 nm)	PEG-poly(l-histidine) (26 nm)	[135]
Melanoma	B16F10 (mouse)	Ce6 (664 nm)	HA (100 nm)	[105]
Multiple myeloma	MM1.S (human)	TiO <sub>2</sub>	Radionuclide ( <sup>89</sup> Zr) coated with transferrin (122 nm)	[136]
Oral	KB (human)	Ce6 (664 nm)	Folic acid-conjugated PEG (100 nm)	[137]
		Tetraphenylporphyrin (469 nm)	Polyfluorene derivative (28 nm)	[138]
	UM1 (human)	5-Aminolevulinic acid (420 nm)	Sulfur-doped carbon dots (28 nm)	[139]
Prostate	PC-3 (human); RM-1 (mouse)	Ce6 (664 nm)	Hydrogel (68.6 nm)	[140]
Squamous carcinoma	SCC7 (mouse)	Pyropheophorbide-a (655 nm)	Polycaprolactone and pluronic F-68 (20 nm)	[123]
	YD-38 (human)	Ce6 (664 nm)	Folic acid-conjugated PEG (200 nm)	[138]

therapy's efficiency, it has become increasingly important to note the fact that a given cell dies is indispensable, but also how those cells are dying is fundamental to the development of strategies to overcome cancer resistance [152]. At the cellular level, PDT has been shown to induce multiple cell death subroutines that can be accidental or regulated (RCD) [11, 153]. Accidental cell death is an uncontrollable form of death, associated to physical disassembly of the plasma membrane caused by extreme physical, chemical, or mechanical cues [154]. On the other hand, RCD results from the activation of one or more signal transduction modules, and hence can be pharmacologically or genetically modulated, at least to some extent [154]. The RCD subroutines already related with PDT include apoptosis and different mechanisms of regulated necrosis, such as necroptosis, lysosome-dependent cell death and ferroptosis [11]. Indeed, the question that has guided a considerable part of cancer research is: has this therapy potential to activate more than one regulated cell death pathway? The design NPS could then be conceived to trigger one or more RCD pathways that the tumor is not able to evade [155, 156].

### 5.3. *In vitro* and *in vivo* studies of NPS efficacy

At the cellular level, the efficacy NPS in PDT should be investigated as a measure of the cell death by using specific techniques based on the evaluation of the cellular plasma membrane rupture such as the use of plasma membrane impermeable probes, like propidium iodide [154]. It can be easily observed that, most frequently, instead of analyzing specific cell death parameters, researchers analyzed cell viability by using approaches based on the evaluation of the metabolic state of the cell measured by tetrazolium dye-based assays, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays [97, 115, 123, 129, 157]. Since these metabolic changes not necessarily reflect the number of death cells in a given context, these colorimetric approaches constitute a only a preliminary form of cell death assessment [158]. Their validity needs always to be linked [159, 160] to the establishment of a robust direct correlation with *bona fide* cell death assays (a deeper information on high-throughput screenings to accurately measure cell death can be assessed in [159, 160]).

Additionally, 2D cell cultures are the models most frequently used for *in vitro* studies [99, 161–163]. The use of monolayer cell culture models promote alterations in the cellular and extracellular environments interactions, and consequently changes in the intrinsic cell morphology, polarity, and method of division [164, 165]. Given these alterations, 3D cultures or organoids have been used to provide a cellular environment similar to the one observed in the *in vivo* TME [166]. Considering cell-cell as well as cell-extracellular matrix (ECM) interactions are quite important because cells that cannot keep an appropriated intercellular and cell-ECM interaction might be artificially more prone to undergo death [167, 168]. In the context of PDT, the selectivity of the treatment was already shown to be higher in 3D than in 2D cultures of breast tumor cells [141]. These cell culture approaches offer a platform of high-throughput model systems able to access important aspects of tumor biology, nanoparticle penetration, treatment regimens and enable to predict the impact of microenvironmental variables on PS dynamics and treatment outcomes [1, 169, 170]. In this scenario, the use of 3D cultures could be explored to provide not only insights regarding the optimization of NPS treatments but also to unveil how this nanostructures act in a more complex environment. It has been shown that the use of organoid culture derived from pancreatic ductal adenocarcinoma is an efficient *in vitro* platform to recapitulate protein expression signature and morphology of patient derived xenografts (PDX). This illustrates one of the advantages of using these models in preclinical studies to predict *in vivo* efficiency of a therapy [171]. This kind of information needs to be taken into account when designing NPSs targeting tumors ECM. These types of particles could then lead to more efficient therapeutic strategies.

Regarding *in vivo* models, animal models used in the NPS studies varies from mice, rats [172], and even rabbits and fertilized eggs [173], where syngeneic and human xenograft tumors as well as genetically-engineered mouse models has been employed as useful tools to explore the impact of the PDT treatment. Even though, mice have been the most used animal in NPS field where the use of tumor subcutaneous injection (TSCI) of syngeneic and xenograft tumors are widely used. TSCI present some limitation once this technique does not ensure the tumor will be implanted in the original organ. As a result, it cannot properly simulate the expected behavior of the tumor or their metastatic properties. The use of orthotopically syngeneic implanted tumor models needs to be considered to overcome this limitation. Despite the disadvantages inherent of any model, this process is more efficient and mimics better what happens in human metastasis [174]. However one needs to highlight that the use of PDX models involve the use of immunocompromised mouse models, completely limiting the study of pro-inflammatory responses triggered by the therapy being tested. Additionally, the efficiency of NPS is in general assessed by the analysis of animal survival curves after PDT and the measurement of tumor volume [103, 115] and weight [113]. Often the studies lack the control comparison of the PS without being loaded to nanoparticles to compare whether there is a real gain of performance of the approach using nanomaterials.

### 5.4. Alternatives for deep-PDT

In addition to the above mentioned use of UCNPs, the nanotechnology for development of TP activation-based strategies have been used to circumvent the limitations of current PDT for deep tumor treatment. An approach that currently deserves attention TP-PDT. Taken into account the spatial and temporal selectivity of the TP activated drug, PDT is highly promising for this future medical trend. As conventional PDT, TP-PDT also enables the addition of the imaging modality to the therapeutic compound. In this field, nanotechnology can have a big impact since theranostic agents based on nanomaterials offer a platform to combine large amounts of drugs with imaging and targeting agents for an improved therapeutic effect [22, 175, 176]. Even when more pre-clinical and clinical evidences are still needed, TP-PDT holds promise as a gentle treatment that needs highly targeted light delivery to limit the damage to surrounding

healthy tissues, especially in the treatment of brain, breast and lung tumors [177, 178]. Indeed, the transition rate for the TP absorption is low and proportional to the square of the light intensity used for excitation. Thus, the TP process requires very high photon density to be detected, provided generally by pulsed lasers [179]. It is important to note that the advantages of pulsed lasers in PDT are still being under investigation [180]. Nevertheless, before considering clinical tests, a complete biodistribution and pharmacokinetics evaluation should be performed. Another factor that needs consideration regarding clinical application of TP-PDT is the fact that TP relies on the recently developed endoscopes equipped with optic fibers [181]. Only practice and time would tell how these instruments could be coupled to TP-PDT treatment of solid tumors.

The versatility in light delivery recently enabled by wireless photonic approaches that allow the possibility of PDT in which miniaturized implantable devices deliver controlled doses of light by wireless powering through thick tissue extends the spatial and temporal precision of PDT to regions deep within the body [182]. In view of the promising results reported with this approach showing targeted cancer therapy by activating light-sensitive drugs deep in the body and suppressing tumor activity *in vivo* in animal models, the combination with the NPS certainly deserve more interest in the PDT field.

### 5.5. Combined therapies

Since the efficacy of conventional cancer therapies are often limited by a dose-dependent toxicity and/or drug resistance PCI may represent a rational and promising approach for combined PDT, chemotherapeutic targeting and killing of drug-therapy in a unique nanoparticle for multi-therapy-resistant cancer cells [183–186]. This field certainly appears very promising in order to tackle drug resistance, which is one of the major challenges in cancer treatment. Moreover, even when a considerable amount of biomarkers to specific tumors have been reported along the years, research still need to be conducted in search for new targets in cancers including gene modulation, elements of the TME, and cellular response pathways, which would contribute with the design of novel drug and combinatory therapies.

As biomedical optics technologies continue to produce light sources with increased power as well as the capability for the use of multiple fibers and decreased size and costs, interest in phototherapies is expected to remain high. Furthermore, next-generation and nanoscale photosensitizing agents have produced impressive preclinical results, but with limited clinical translation to date. Ironically, the advanced targeting and activation features of these agents might lead to manufacturing complexities that impairs their clinical translation. Nevertheless, antibody-targeted PDT, or photoimmunotherapy, is under investigation in large-cohort clinical trials and holds potential to move forward as a next-generation technology [34].

## 6. Conclusions and final remarks

Considering the information available one can conclude that the potential advantage of nanotechnology is the possibility of loading higher amounts of PS bypassing the major problems of water solubility and *in vivo* photoactivation. On top of this, nanoparticles can be functionalized with multiple targeting moieties, such as antibodies or peptides, improving selectivity of targeted delivery. Moreover, we have presented different formulations possibilities, including biodegradable polymers and metallic nanoparticles displaying also magnetic properties. As well as hybrid nanoparticles, the development of magnetic NPS can enhance localization to the tumor and also allow another therapeutic strategy besides PDT, such as imaging techniques. By adding the imaging modality to the therapeutic agent, these NPS agents enable an accurate diagnosis and are capable of optimize the therapeutic protocol and outcome.

The importance of the photoactivity of nanotherapeutics is not only restricted to their use in phototherapies, but also encompasses multiple parameters that distinguish such photonanomedicine formulations from the emerging pool of therapeutics. The design of novel nanoformulations for PDT must incorporate a mechanistic insight into the photochemical activity within a controlled cellular system, evaluation of subcellular localizations, establishment of sites of photodamage and the mechanisms of cell death to better predict their potency and uncover fit points. It is also essential to note that insights into the molecular mechanisms for PDT may not hold for different cell types, neither different culture methods (e.g. 3D models) and *in vivo* [52].

The most serious limitation in promoting nanotechnology applied to PDT as a frontline anticancer therapy is related to the fact that large, controlled, comparative, randomized clinical trials either have not been undertaken yet or could not prove a significant advantage over conventional approaches. The latter result might correspond to the fact that PDT has been and is still mostly tested on patients with advanced cancers that are refractory to other therapies. This implies that possible positive effects of NPS on a local level cannot be honored adequately. Therefore, a considerable amount of information regarding the use of NPS is reported from *in vitro* studies. It is important to note that when *in vitro* research is done on NPS, the interaction between NPS, biological systems, and the immune system *in vivo* is somewhat overlooked. Finally,



a careful evaluation of the potential clinical fields for nanomaterials developed for PDT will contribute to reinforce the concept that 'PDT is not only a class of drugs, but a fascinating innovative concept' [187].

## Data availability statement

No new data were created or analyzed in this study.

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## Authors' contributions

Responsible for the paper: Labriola L.

Concept, design, definition of intellectual content: dos Santos A F, Labriola L.

Literature search, manuscript preparation and revision: dos Santos A F, Arini G S, de Almeida D R Q, Labriola L.

Manuscript editing: dos Santos A F, Labriola L.

## Conflict of interest

All authors declared that there are no conflicts of interest.

## ORCID iDs

Ancély Ferreira dos Santos  <https://orcid.org/0000-0003-4985-6187>

Leticia Labriola  <https://orcid.org/0000-0003-3899-6088>

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