Published in final edited form as:

J Biomed Nanotechnol. 2014 September 1; 10(9): 1937–1952. doi:10.1166/jbn.2014.1953.

Photodynamic Therapy: One Step Ahead with Self-Assembled Nanoparticles

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Abstract

Photodynamic therapy (PDT) is a promising treatment modality for cancer with possible advantages over current treatment alternatives. It involves combination of light and a photosensitizer (PS), which is activated by absorption of specific wavelength light and creates local tissue damage through generation of reactive oxygen species (ROS) that induce a cascade of cellular and molecular events. However, as of today, PDT is still in need of improvement and nanotechnology may play a role. PDT frequently employs PS with molecular structures that are highly hydrophobic, water insoluble and prone to aggregation. Aggregation of PS leads to reduced ROS generation and thus lowers the PDT activity. Some PS such as 5-aminolevulinic acid (ALA) cannot penetrate through the stratum corneum of the skin and systemic administration is not an option due to frequently encountered side effects. Therefore PS are often encapsulated or conjugated in/on nano-drug delivery vehicles to allow them to be better taken up by cells and to more selectively deliver them to tumors or other target tissues. Several nano-drug delivery vehicles including liposomes, fullerosomes and nanocells have been tested and reviewed. Here we cover non-liposomal self-assembled nanoparticles consisting of polymeric micelles including block co-polymers, polymeric micelles, dendrimers and porphysomes.

Keywords

Cancer; Photodynamic Therapy; Self Assembly; Dendrimers; Block Copolymers; Nanoparticles; Micelles

INTRODUCTION

Photodynamic Therapy

Photodynamic therapy (PDT) is a promising non-invasive localized treatment modality for a diverse range of diseases, including various types of cancers, infections, and inflammatory conditions.¹⁻⁴ PDT combines photoactivatable fluorophore, which is called photosensitizer (PS), light and reactive oxygen species (ROS), which lead to cascade of reactions to result in an apoptotic or necrotic response in malignant cells. Broadly, PDT starts with topical or intravenous administration of the PS to the body. Following the introduction of the PS, a certain time interval is allowed for the PS to circulate and preferentially localize in the target lesion. It should be noted that the optimal period for selective PS accumulation varies according to the PS used. For instance, while a 'vascular' PS localizes in the tumor within minutes, a 'cellular' PS takes hours or even days.^{4, 5} Following the localization, the tumor site is irradiated with visible (usually red) light at a wavelength(s) that is complimentary to the absorption spectrum of the PS.^{6, 7} Upon irradiation, the PS in its ground singlet state (S_0) absorbs a photon of light which excites an electron to a higher state PS (S_1).⁶ The PS in its short-lived excited singlet state (S_1) can return to its ground state via different pathways including fluorescence (emission of a photon) or non-radiative decay (internal conversion) producing heat. An alternative pathway is that the PS can undergo the non-radiative process of inter-system crossing to give the excited triplet state molecule. Since the direct decay of the triplet to singlet state, losing energy by photon emission (phosphorescence), is forbidden, this transition is relatively slow. Therefore, the triplet state sensitizer has a long lifetime that allows it to react with its chemical environment. This involves the transfer of energy to molecules in close proximity via two different ways known as type I and type II reaction processes. In type I reaction, free radicals, formed by either hydrogen or the electron transfer from the PS, react with oxygen, and generate ROS such as superoxide anion, hydroxyl radical and hydrogen peroxide. Whereas, in the type II reaction process, the PS in the excited triplet state directly transfers energy to molecular oxygen (which also is a triplet in its ground state) to form the excited singlet state oxygen which is a highly reactive oxidizing agent. ROS generated as a result of these reactions lead to cell death mainly through apoptosis or autophagy.⁸ In PDT, it is often difficult to distinguish between two mechanisms. There is probably a contribution from both Type I and Type II mechanisms. After singlet oxygen production, the PS can return to its starting point where it is available to start the whole process again. Individual PS molecules may do this cycling process thousands of times before they are destroyed by photobleaching.

PDT offers several advantages over other cancer therapies. First of all, it is considered a minimally invasive treatment with minimal side effects. It is usually well tolerated by the patients and can be repeated several times without interfering with other treatment options. Due to the desirable rapid clearance time of the PS from the healthy tissue, the toxicity of the PS is limited to the lesion that receives illumination. The dual selectivity (localization and photoactivation), makes PDT a more favorable treatment modality compared to chemotherapy and radiotherapy with several systemic side effects. It has previously been shown that cancer tissues tend to accumulate and retain PS to a greater extent than normal tissues, which was attributed to the enhanced microvasculature permeability and impaired

lymphatic drainage in the tumor tissue, the so called enhanced permeability and retention (EPR) effect.^{9–11} It has also been suggested that PS can bind to low-density lipoproteins (LDL) in the serum and the fact that some tumor cells overexpress the LDL receptors on their surface may facilitate PS selective uptake.¹² Lastly, focusing the light source solely on the tumor site further enhances tumor-specificity.

Due to the non-scarring nature of PDT, 5-aminolevulinic acid (ALA)-PDT (Fig. 1) and methyl ALA (MAL)-PDT (Fig. 1) are rapidly becoming preferred techniques in the clinic for treating actinic keratoses, basal cell carcinoma and squamous cell carcinoma.¹³ In the last decade, it has also been clinically investigated for its use in precancerous and dysplastic conditions that occur in the cervix, vulva, and perianal region.¹⁴ Moreover, several PS have been clinically approved or are in clinical trials for treatment of other types of cancers such as bladder cancer, lung cancer, prostate cancer, esophageal cancer and head and neck cancer.²

A Step Toward Personalized Therapy

Theranostics is a new development in biomedicine that combines diagnostic and therapeutic functions into a single agent with the goal to develop personalized treatment.¹⁵ While the “diagnostic” part of the system frequently involves imaging modalities based on fluorescence, PET, radiosotopes, MRI or ultrasound reporters, the “therapeutic” part can involve cytotoxic drugs, radioisotopes, gene therapy vectors or light-activated drugs.¹⁶ Use of PS in medicine is not restricted to the therapeutic generation of singlet oxygen and ROS. Many PS are bright fluorophores, they tend to emit in the near infrared (NIR) region of the electromagnetic spectrum that is beneficial for *in vivo* imaging. Therefore, PS can be used as theranostic agents. A fluorescent PS can be used for determining the optimal treatment parameters before starting the treatment with PDT.⁶ Fluorescence imaging can aid in confirming PS localization and measuring the degree of uptake by the diseased tissue. Once the malignant cells uptake the PS, the target site emits fluorescence to provide visible guidelines for the therapy. Moreover, the fluorescence intensity of a PS may differentiate normal and malignant regions, acting as an image-guidance tool. Fluorescent signatures may also be used as an optical histopathology that enables distinguishing between benign and malignant tissues, thus avoiding the invasive biopsy procedures. In addition, evaluation of the success or failure of treatment may be monitored through the PS fluorescence (as target tissue is destroyed, the fluorescence signal decreases) which may be a guide for real-time adjustments during therapy.

NANOPARTICLES AS DRUG DELIVERY VEHICLES FOR HIGH EFFICACY PDT

As mentioned earlier, PDT has various advantages over existing cancer treatments. In chemotherapy, aside from the systemic toxicity, resistance is frequently encountered due to specific tumor environment and several molecular mechanisms such as over expression of efflux transporters.¹⁷ Systemic toxicity and complications are also significant concerns in radiation therapy as well as in surgery, which is an invasive procedure on its own.^{18, 19} Although PDT seems to have the potential to overcome these challenges, the current PS and

light sources still have a number of limitations and have room for improvement. Skin phototoxicity (patients treated with PDT must avoid direct sunlight or strong indoor lighting for weeks),²⁰ low tumor/normal tissue accumulation ratio (especially in organs such as liver and spleen which possess leaky vasculature),²¹ strong oxygen dependence which cannot be well satisfied in hypoxic tumor tissue,²² sub-optimal EPR effect, aggregation of hydrophobic PS resulting in reduced ROS formation due to self-quenching of the excited state,^{23, 24} and limited penetration of light to deep tissues are among the main challenges.

Several nanoparticles (NP) including (but not limited to) liposomes, dendrimers, pH sensitive polymers and fullerenes have recently attracted attention as PS carriers. These NPs offer great hope for overcoming some of the afore-mentioned limitations and moving PDT forward in another direction. However several factors need to be taken into consideration in order to optimize the choice of NP:

1. increased structural stability thus being resistant to degradation in different biological fluids, while having a long circulation time in blood;^{25–28}
2. Optimal size-large enough to escape renal excretion (15–30 nm) but at the same time, small enough to extravasate and accumulate at the tumor site, so called passive targeting;²⁷
3. good thermodynamic and kinetic stability;²⁹
4. long shelf-life;^{27, 28}
5. high drug-loading capacity, good biocompatibility and reduced systemic toxicity;³⁰
6. ability to provide anchoring site for tumor specific ligands or antibodies for recognition and specific binding (active targeting);³¹
7. responsiveness to stimuli such that once the NP is at the target site, the drug should be released. Although nanosized drug delivery vehicles have attracted widespread attention as PS carriers, some major drawbacks of vehicles such as liposomes are their short plasma-half life which leads to insufficient time for tumor cell uptake, rapid degradation and elimination by the reticuloendothelial system (RES) resulting in accumulation in liver and spleen.³²

Consequently, they are unable to achieve sustained drug delivery over a prolonged period of time.³³

Most PS are aromatic compounds with large delocalized π electron systems (e.g., Porphyrin, chlorin, bacteriochlorin or phthalocyanine rings as shown in Fig. 1), which allow them to achieve high quantum yield for singlet oxygen formation and to absorb energy in the IR or NIR region (> 600 nm) of the electromagnetic spectrum.³⁴ On the other hand, due to the strong π - π interactions between the two aromatic cores of neighboring molecules, means the PSs form dimers and higher order aggregates both in organic solvents and especially in aqueous media. This aggregate formation leads to significant reduction in ROS formation due to self-quenching of the excited state.³⁴ These properties may also prevent encapsulation into NP such as liposomes and PS encapsulated into NP may induce aggregation of PS.^{34, 35}

DRUG DELIVERY VEHICLES FOR PDT

Molecular self-assembly is ubiquitous in nature; cell membranes are formed by self-assembly of phospholipids and soap bubbles originate from the self-assembly of small-molecule surfactants.³⁶ These molecules are composed of one or more hydrophobic tails and a hydrophilic head group.³⁶ Self-assembled materials are ideal as theranostics because they can encapsulate both imaging and therapeutic agents whose biomedical utility had previously been limited by inadequate aqueous solubility.³⁷ For decades, self-assembled vesicles have been used for sequestering high concentrations of hydrophilic compounds,³⁸ and for controlling their temporal release as well as their distribution to achieve maximal therapeutic efficacy.³⁹ More recently, amphiphilic peptides and polymers have been shown to spontaneously form a variety of morphologies, which include but are not limited to spherical micelles, cylindrical micelles, bicontinuous structures, lamellae and vesicles^{36, 40, 41} and serve as useful nanocontainers in aqueous solution.⁴² Also supramolecular self-assembly enables the efficient and high-throughput fabrication of these complex multicomponent nanostructures.^{43–45}

POLYMER CARRIERS

Block Copolymers

Recent developments in polymer synthesis have given rise to diverse controlled polymerization techniques such as anionic or living radical polymerization, for preparation of polymers of various architectures, including linear block copolymers (BCP) (Fig. 2), graft copolymers (Fig. 2), dendritic polymers, cyclic polymers and star-like polymers.²⁵ It has been shown that all these types of polymers of different structures have the ability to self-organize into aggregates of diverse morphologies under certain conditions.³⁶ For instance, BCP have the same basic structure as lipids (hydrophobic head and hydrophilic tail) but consist of chemically distinct polymer chains, which are covalently linked in a series of two or more. A defining property of amphiphilic BCP is the ability of individual block copolymer molecules, termed “unimers,” to self-assemble into micelles in aqueous solutions, to minimize energetically unfavorable interactions between hydrophobic drugs and water.⁴⁶ More than 20 different structures can be achieved through thermodynamic or kinetic variation. As an example, working with polystyrene-*b*-poly(acrylic acid) (PS-*b*-PAA) under different conditions, it is possible to achieve a wide range of thermodynamically controlled morphologies such as spherical micelles, rods (cylindrical micelles) and bilayers (lamellae and vesicles).

Spherical micelles are composed of a spherical core surrounded by coronal chains. While the hydrophilic coronas afford the spherical micelles' solubility in water, hydrophobic cores provide an ideal location for encapsulation of hydrophobic PS. Moreover, they have been developed beyond simple core-shell particles, polyionic complex micelles (PIC) being one example where oppositely charged polymers form PIC through electrostatic interaction in aqueous solution^{47, 25} (Fig. 3). For example, depending on the type of amino acid, poly(ethylene oxide)-block-poly(*L*-aminoacid) (PEO-*b*-PLAA) BCP may be positively or negatively charged at their side chains. Macromolecules with an opposite charge can form PIC by combining with the PLAA segment of the BCP, thus neutralizing the charge and

satisfy the required amphiphilicity for micellization of the complex.²⁸ Zhang et al. reported that upon mixing aqueous solutions of a polycationic porphyrin dendrimer and a polyanionic PEG-poly(aspartic acid) BCP (PEG-*b*-PAA), highly stable micelles were formed as a result of electrostatic interactions and hydrogen between the two components.⁴⁸

Recent developments indicate that certain polymer NP can function not only as a drug carrier but also can function as biological response modifiers.^{49, 50} One example of such materials is Pluronic® BCP (poloxamers) that can cause various functional alterations in cells. They are non-ionic triblock copolymers composed of a hydrophobic poly(propylene)oxide core terminated by two hydrophilic poly(ethyleneoxide) chains on both ends (therefore, often abbreviated as PEO-PPO-PEO). They can readily form self-assembled structures due to their amphiphilic properties.⁵⁰ Incorporation of drugs/PS into the core of the micelles that are formed by self assembly of Pluronic BCP results in increased solubility, metabolic stability and circulation time for the drug.⁵⁰ Moreover, single molecular chains of the copolymer unimers, induce immune responses by acting as an adjuvant,^{52, 53} cause intracellular ATP depletion^{54, 55} and inhibit the activity of drug efflux transporters which are held responsible for multiple drug resistance.^{22, 50, 56–58} They were also shown to alter apoptotic signal transduction, another way of overcoming multi drug resistance. One example of use of Pluronic BCP is to combine it with the chemotherapy drug doxorubicin.⁵⁹ Treatment of cancer cells with doxorubicin alone activates proapoptotic signaling however it also activates the antiapoptotic cellular defense.⁶⁰ In contrast, the treatment of the cells with doxorubicin/Pluronic BCP P85 formulation was shown to significantly enhance the proapoptotic activity of the drug without the disadvantage of activating the antiapoptotic cellular defense.⁶⁰ Similarly, up-regulation of the proapoptotic genes (p53, p21, and Bax) and down-regulation of antiapoptotic gene (Bcl-2) by conjugates of linoleic acid and Pluronic F127 was reported by Guo et al.⁶¹

Glutathione, a ubiquitous tripeptide, protects cells against ROS, various toxins, mutagens, and drugs. Batrakova and colleagues demonstrated that Pluronic P85 decreased glutathione levels and caused inhibition of glutathione-S-transferase activity in cells, which in turn reduce efficacy of the glutathion/glutathione-S-transferase drug detoxification system.⁵⁵ The mechanisms of resistance to PDT may be shared with the general mechanisms of multi-drug resistance. Efficacy of PDT, especially against multi-drug resistant tumors can be significantly improved by combination with Pluronic BCP.^{22, 51, 58, 62–64} However, to this point, reports in the literature are still scarce and mainly limited to its effects on PS delivery.

In an aqueous medium, amphiphilic block copolymer micelles self-assemble to form nano-sized micelles such that the hydrophobic region stays in the micelle-center, which is surrounded by a shell of hydrophilic polymer. While the hydrophilic shell acts as an interface between the micellar core and the surroundings, the hydrophobic core can serve as an excellent loading carrier for hydrophobic agents such as PS for PDT. Their small size enables more efficient accumulation and retention (EPR effect is limited to carriers of around 100 nm in size)⁶⁵ since large NP (diameter > 200 nm)⁶⁶ are easily eliminated by opsonization and phagocytosis by macrophages.⁶⁷ Thermodynamic and kinetic stability, high drug-loading capacity and good biocompatibility also make them a favorable alternative relative to liposomes.³⁰

Polymeric Micelles

Micelles can be co-assembled with unique molecules that can allow the targeted delivery of the PS to the tumor tissue. It is possible to accomplish the guided delivery of the PS by a variety of means including magnetic or ligand-directed methods.⁶⁸ In addition, the controlled release of drugs in response to environmental cues such as pH can be achieved by using micelles composed of stimuli-sensitive copolymers. An example of inducible drug delivery vehicles is that of pH sensitive micelles which are composed of poly(2-ethyl-2-oxazoline)-*b*-poly(*D,L*-lactide) (PEOz-*b*-PLA) diblock copolymers. This vehicle was used as a carrier for meta-tetra(hydroxyphenyl)chlorin (m-THPC) (Fig. 4(a)), a second-generation clinically approved PS in Europe.^{69, 70} Although m-THPC is less phototoxic to the skin than the first generation of PS, patients receiving m-THPC must still avoid direct sunlight for more than 2 weeks.⁷¹ The PS was expected to be protected by micelles while circulating in the blood and to be released from their carriers only when in the cytosol of the cell (polymeric micelles are taken up by cells via endocytosis and trapped in acidic endosomal/lysosomal compartments). m-THPC loaded micelles demonstrated significantly reduced the skin phototoxicity compared to free m-THPC while maintaining its tumor inhibition activity.⁷⁰ However no apparent difference was observed between free m-THPC and the m-THPC-loaded micelles with respect to tumor accumulation and PDT efficacy.⁷⁰

In order to improve the PDT efficacy of the m-THPC-loaded micelles, Syu et al. developed folate-conjugated m-THPC-loaded micelles.⁷² Folate receptor, a well-known cancer-associated protein, internalizes bound folic acid through endocytosis, a process that can be utilized to target folate-conjugated NP.^{69, 73, 74} It was shown that m-THPC-loaded folate-targeted micelles were taken up and accumulated in KB cells, which overexpress the folate receptor, *in vitro*, and in tumors *in vivo*.⁷² In this study, in addition to reduced skin photosensitivity, a significant increase in antitumor efficacy was observed with the folate-micellar formulation.⁷² Inhibition of cell proliferation was accompanied by reduction of vessel density, while the folate-targeted micelles gave the largest vascular damage.⁷² Another interesting finding was that cellular uptake of the folate-conjugated micelles could be significantly inhibited after folate pretreatment. This is probably due to competitive inhibition; such that excess free folate in the medium prevents uptake of folate-conjugated m-THPC-loaded micelles into KB cells by competitively binding to folate receptors on the cell surface.⁷²

Peng et al. investigated a possible synergistic effect by designing a functionalized micellar delivery system that could serve as a dual carrier for hydrophobic PDT and chemotherapy agents.⁷⁵ Methoxypoly(ethylene glycol) (mPEG) and poly(epsilon-caprolactone) (PCL) were employed to synthesize amphiphilic 4-armed star-shaped chlorin-core diblock copolymers that self-assembled to form micelle-like structures. For the combination study, a chemotherapeutic agent, paclitaxel, was trapped in the hydrophobic inner core of micelles.⁷⁵ As expected, these micelles improved the cytotoxicity of paclitaxel significantly in MCF-7 breast cancer cells following irradiation of PDT agent.⁷⁵

Another interesting phenomenon that was recently reported was termed a “photoactivation switch” of PS from the type II to type I photochemical mechanism via the aid of an electron

rich micelle core serving as an electron reservoir.⁷⁶ As mentioned earlier, while in type I mechanism hydroxyl radicals, hydrogen peroxide and other ROS are generated, in type II mechanism singlet oxygen ($^1\text{O}_2$) is produced. Generation of $^1\text{O}_2$ is highly dependent on the concentration of molecular oxygen in the tissue,⁵⁸ and microenvironment of the tumors, which is usually hypoxic.⁷⁷ In addition to this consideration, vascular damage and the photochemical oxygen consumption during PDT lead to an even more severe shortage of oxygen.^{58, 78, 79} In order to overcome this challenge, Ding and colleagues encapsulated 5,10,15,20-tetrakis(meso-hydroxyphenyl)porphyrin (mTHPP) (Fig. 4(b)) in the core of electron-rich PEG-*b*-poly(2-(diisopropylamino) ethyl methacrylate) (PEG-*b*-PDPA) and achieved highly increased production of type I radical species compared to encapsulation with electron-deficient PEG-*b*-poly(*D,L*-lactide) (PEG-*b*-PLA) micelle.⁷⁶ With the presence of the electron-donating PDPA segment, the generation of $\text{O}_2^{\bullet-}$ through the electron transfer pathway competes with $^1\text{O}_2$ production through the energy transfer process under aerobic environments, and becomes dominant under hypoxic conditions. This new method resulted in an amplified PDT response, especially under low oxygen conditions.^{76, 80, 81}

While lamellae are either flat or slightly curved bilayers, vesicles are completely closed bilayers. Vesicles can be defined as hollow spheres with a continuous bilayer wall surrounded by internal and external coronas. Due to their high thermodynamic stability they are more frequently used than lamellae.³⁶ BCP vesicles, known as ‘polymersomes’ hydrophobic wall and hydrophilic internal and external coronas make them promising candidate for delivery of PS to the tumor site. BCPs are sometimes called ‘super amphiphiles’ due to their high molecular weights which is several orders of magnitude bigger than those of small-molecule amphiphiles such as phospholipids. BCP vesicles have many properties of liposomes (lipid based vesicles) and they offer several advantages over liposomes:

1. they are more stable;
2. they have improved mechanical properties;
3. their higher molecular weight lead to increased wall thickness;
4. as a result of thick wall accompanied by an increased bending rigidity (with a surprisingly similar membrane elasticity to phospholipid vesicles), they exhibit reduced permeability with minimal leakiness;
5. their thick wall serves as a physical barrier to protect encapsulated material from outside environment;
6. they have ability to withstand higher tension and area strain before rupture.^{36, 46, 82}

Polyethylene glycol (PEG) is extensively used for drug delivery with unique characteristics such as absence of immunogenicity and toxicity, and high solubility in both in aqueous and organic solvents.⁸³ PEG derivatives are also more stable toward degradative enzymes and have increased molecular weight, resulting in a prolonged half-life.⁸³ Moreover, PEG conjugation increases the ability of the chemotherapy agent to penetrate tumor cell membranes.⁸⁴ PEG-modified lipid vesicles have already shown considerable utility in delaying vesicle clearance from the circulation, However PEG-lipids tend to be micelle-

forming amphiphiles, and the vesicles can stably incorporate only a relatively small amount of PEG-lipid (mole fraction up to 11%) into a lipid membrane.⁸⁵ According to Bradley et al.'s study, only 4–6% of PEG-lipid is stable in liposomes, and upon serum incubation, liposomes immediately lose about 1/3 of their PEG-lipids, presumably due in part to facilitated micellization.⁸⁶ Polymersomes, in contrast, are 100% PEGylated and overcome the instability of PEG-lipid vesicles by increasing the hydrophobic volume fraction of the amphiphile together with the hydrophilic PEG volume fraction.⁸⁵ Vesicles with poly(ethylene oxide) (PEO) as coronas are biocompatible and do not interact with the immune system, due to the very limited interaction of the hydrated and non-ionic PEO blocks with proteins.³⁶ This so-called “stealth” ability provides increased circulation time of the vesicles.

By altering the molecular weight and the chemical structure of the amphiphilic polymer, different blocks can be functionalized in order to produce ‘smart’ NP.^{87, 88} For instance, polymersomes with proteins decorated at the ends of the chains were designed for targeted binding, while polymersomes made of tailored copolymers such as pH- or oxidation-sensitive hydrophobic blocks, which respond to external stimuli, can be used for controlled drug release.^{87, 88} In a similar fashion, photoresponsive polymersomes have been designed where light (e.g., UV) delivery can induce destruction of vesicles that contain photoreactive groups.⁸⁹ Mabrouk et al. investigated the UV photo-response of polymersome-Ce6, a photo-inert polyethyleneoxide-*b*-polybutadienediblock copolymer (PEO-*b*-PBD) loaded with the amphiphilic PS chlorin(e6)–(Ce6) (Fig. 4(c)).⁹⁰ Different parameters affecting the photo-response, including osmotic swelling, membrane cross-linking and vesicle deflation were quantitatively monitored in the study. The group concluded that depending on the Ce6 concentration, polymer vesicles could significantly swell due to the osmotic pressure which was built up following photooxidation of the Ce6-loaded membrane and the vesicle interior.⁹⁰ Longer illumination times resulted in membrane cross-linking. Composite Ce6-copolymer vesicles showed potential as a new class of light-sensitive drug carrier however limitations exist. For instance, synthesis of polymersomes involve complicated and time-consuming procedures such as double-emulsion and a size extrusion process which are performed to achieve uniform vesicles.³³ With an attempt to overcome these drawbacks, Hsu et al. designed an easily prepared, robust and uniform NP platform (porphysomes) that consist of 4-armed porphyrin-polylactide (PPLA) conjugates.³³ Owing to their unique structure, these NP were suggested to have good passive tumor targeting ability, higher stability in aqueous solutions, greater ability to generate ¹O₂ upon irradiation in aqueous solution, and were suggested to cause less photosensitivity if used in PDT.³³ Moreover, the fluorescence intensity was almost insensitive to the environmental pH which was also supported by the observation that no self-quenching in acidic environments such as tumor regions or intracellular endosomes/lysosomes was reported.³³ The authors showed that when HeLa cells were incubated with the porphysomes followed by light, membrane blebbing was observed. The group also suggested that by replacing the inner core porphyrins of PPLA by other PS with longer excitation wavelengths such as chlorin (Fig. 1),⁷⁵ phthalocyanine (Fig. 1)⁹¹ or NIR two-photon absorbing porphyrin dimers,⁹² it is possible to achieve an increased light penetration. Additionally, these conjugates could be used as contrast agents for ultrasound imaging by providing a better alternative to lipid-based micro/nanobubbles. Their

robust and highly stable polymer based composition allowed superior air/gas encapsulation suggesting they could have a role in cancer theranostics.^{33, 93}

Dendrimers

Dendrimers are monodisperse, globular macromolecules with a large number of peripheral groups. Two different strategies can be employed for the synthesis of dendrimers. They can be formed either by growth outwards from a central core (divergent method) or they can be synthesized from the periphery inwards, terminating at the core (convergent method).⁹⁴ The amount of branching is described by the generation number. The central core molecule with sites that will become branched is called generation 0 (G0) and each successive addition of new branched points define dendrimer's generation (e.g., G1, G2, G3). Dendrimers are promising candidates for improved drug delivery because unlike conventional polymers, dendrimers are synthesized by a stepwise procedure, which in turn results in a highly regular branching pattern and well-defined architecture and molecular weight. Consequently, dendrimers have a strictly controlled number of functional groups on their periphery for conjugation with bioactive molecules. These terminal groups may further be used to provide solubility, targeting (targeting moieties can be attached to dendrimer surface and enable more specific tumor targeting through over-expressed receptors in the tumor site), or other moieties to tune the biological properties and control the fate of the drugs. One of the key features of dendrimer-drug conjugates is their ability to achieve a high drug payload via attaching multiple drug molecules to the dendrimer.^{95, 96} Another advantage is that certain dendrimer PS can elicit ROS generation even at very high concentrations, because the dendritic wedges that surround the core of PS, can sterically prevent aggregation of the center dye molecules.^{97, 98} The main principles that are taken into account while designing dendrimer structures are that:

1. Overall charge of the dendrimer molecule-while negatively charged and neutral dendrimers are mostly biocompatible, positively charged species show varying degrees of toxicity;
2. dendrimer architecture-since pharmacokinetics is highly dependent on the structure;
3. degree of PEGylation-as the number of PEG increases per dendrimer molecule, water solubility and size also increase and lead to improved retention and biodistribution;
4. placement of the drug-drugs can either be internalized into the void space between the periphery and the core, or they can be functionally attached to functionalized surface groups.⁹⁴

At present the modified polyamidoamine (PAMAM) has been the most frequently investigated dendrimer, in part because PAMAM G0 to G10 are commercially available with a wide range of peripheral and end groups, and various molecular weights.

Protoporphyrin IX (PpIX) (Fig. 4(d)), an efficient hydrophobic PS in PDT may either be used exogenously (with the requirement for formulation of the PS) or it may be biosynthesized endogenously within the cells through administration of ALA, which is a

naturally occurring precursor in the biosynthetic pathway of heme. Due to limited capacity of ferrochelatase to convert PpIX into heme, the presence of excess exogenous ALA in cells induces accumulation of PpIX.^{99, 100} However, ALA (Fig. 1) penetrates skin lesions poorly upon topical administration and ALA-PDT causes significant pain. When given orally or intravenously, apart from its limited bioavailability, it is associated with nausea, vomiting, decreases in systolic and diastolic pressure as well as transient abnormal liver functions.¹⁰¹ Additionally, cellular uptake of ALA is limited by its hydrophilic nature.¹⁰² Esterified ALA derivatives have been extensively studied as prodrugs,^{103–105} in particular the methyl and hexyl esters, and approval has been granted for treatment of actinic keratosis and basal cell carcinoma using the methyl ester derivative in Europe and Australia. Despite the current progress, further improvements are still required for both PpIX and ALA delivery.

Buhong et al. formulated PpIX with spherical micelles, [methoxy poly(ethylene glycol)-*b*-poly(caprolactone)] diblock copolymers in order to achieve high loading efficiency, increased intracellular uptake, and to avoid use of toxic organic solvents such as DMSO.¹⁰⁶ Tumors exhibit a lower extracellular pH compared to normal tissues because of the high glycolysis rate due to the Warburg effect.¹⁰⁷ Moreover, following intravenous administration, polymeric micelles are taken up by via endocytosis. Subsequently the pH value of the endocytic vesicles gradually decreases due to the protons that are pumped into the lumen of the vesicle during the transition to lysosomes.¹⁰⁸ In an attempt to improve tumor-targeted drug delivery system in PDT, folic acid (FA)-conjugated amphiphilic block copolymers of PEG and poly- β -benzyl-*L*-aspartate (PBLA) were synthesized with the potential to act as pH-responsive PS release reservoirs.¹⁰⁹ 2,4-Diacetyl deuteroporphyrin IX dimethyl ether (DD-PpIX) was conjugated to amphiphilic copolymeric nanoparticles (to produce FA-PEG-P(Asp-Hyd)-DD-PpIX) which formed micelles in aqueous solution. *In vitro* release tests demonstrated strong pH dependence¹⁰⁷ and more than 97% cellular uptake have been achieved using HeLa cells.¹⁰⁹

Several studies investigated the use of dendrons and dendrimers for ALA delivery. The term dendron refers to a monodisperse wedge-shaped section of a dendrimer. Dendrons have only one reactive site for conjugation of drug molecules and have several terminal groups. Rodriguez et al. investigated the efficacy of a trimer of ALA, aminomethane tris-methyl 3m-ALA; a G0-ALA dendron with a free amine at the core and three ALA groups at the periphery (Fig. 4(e)). In LM3 murine mammary adenocarcinoma cells, the dendron induced similar porphyrin levels compared to equal concentrations of free ALA (Fig. 1) and it was taken up with comparable efficiency to free ALA. Upon both systemic and topical administration of the dendron to tumor-bearing mice, higher porphyrin levels were achieved compared to the widely investigated lipophilic hexyl ester derivative in most tissues studied. However, the group reported that only one out of the three ALA molecules was cleaved from the dendron within the cells. Also it was stated that it was not possible to improve upon the PpIX levels induced by ALA *in vivo*.

In order to determine the key factors influencing the efficacy of the dendritic derivatives, Battah et al. investigated the efficacy of a set of G0-ALA dendrons with varying cores and linker lengths, including an amino core (3m-ALA) (Fig. 4(e)) with a methyl linker, a nitro core (3H-ALA) (Fig. 4(f)) with a propyl linker and an aminobenzyloxy carbonyl core (3Bz-

ALA) (Fig. 4(g)) with a methyl linker, each attached to 3 ALA molecules.¹⁰² The study was performed with the transformed PAM 212 keratinocyte cell line and skin explants. While all dendrons induced higher porphyrin production when compared to free ALA *in vitro*, 3H-ALA not only led to almost 10-fold higher porphyrin generation at lower concentrations but also demonstrated the highest phototoxicity. Furthermore, when applied topically to explanted rat skin, 3H-ALA generated higher porphyrin fluorescence compared to 3m-ALA and free ALA. Based on the results, the authors concluded that the presence of lipophilicity and steric hindrance within the dendritic structure, which could restrict access to intracellular esterases for liberation of ALA are one of the key factors influencing the efficacy of the dendritic derivatives.

Two studies investigated macromolecular delivery of 5-ALA based on the assumption that a dendrimer bearing a high ALA 'payload' can deliver a higher quantity of ALA per molecule to the cells.^{96, 110} In one study, using the dendrimer containing 18 ALA residues coupled via ester linkages Nbis{N-[tris(5-aminolaevulinyloxymethyl) methyl]propionamido} propionamido)carbamido]benzene 18 trifluoroacetic acid (18m-ALA), it was shown that at lower concentrations, 18-ALA (Fig. 5(h)) gave superior PpIX production *in vitro* compared to free ALA.⁹⁶ Moreover, PpIX production was sustained up to 24 h using the ALA dendrimer, whereas using free ALA, significantly less PpIX was present following 24 h.⁹⁶ This may suggest that ALA is being released gradually from the internalized dendrimer, thereby resulting in sustained porphyrin production in cells up to 24 h after exposure to the compound. The second study, which was performed earlier with 18m-ALA (Fig. 5(h)) using acetamido linkers, had demonstrated slightly lower efficacy indicating that polyamidoamine linkers allow greater esterase accessibility for cleaving ALA groups from the hyperbranched structure.¹¹⁰ Noteworthy, unlike the G0-ALA dendrimers which underwent active transport and passive diffusion for internalization, macropinocytosis had a key role in the uptake mechanism of 18m-ALA dendrimers.⁹⁶

Ideta and colleagues designed a G3-poly(benzyl ether) dendrimer PIC micelle complex which comprised of 32 negatively-charged carboxylate periphery groups having a zinc porphyrin at the core (Fig. 5(i)). These negative charges allow stable incorporation of the dendrimer into the supramolecular nanocarrier, surrounded by positively charged linear PEG-lysine BCP.¹¹¹ *In vitro* studies of PIC micelle complex with Lewis lung cells demonstrated 280-fold increase in phototoxicity when compared to free dendrimer-based PS which can be attributed to their negatively charged periphery leading to lower cellular uptake.¹¹¹ Based on the encouraging results, the group carried out an *in vivo* study, testing the efficacy of encapsulated dendrimer PEG-lysine micelle system for treatment of choroidal neovascularization (CNV).¹¹¹ Dendrimer-porphyrin loaded micelles achieved a highly selective accumulation in the CNV lesions, and a lower fluence of light was sufficient to occlude the CNV lesions which was attributed to the prevention of porphyrin aggregation by steric hindrance.¹¹¹ Moreover endothelial cells of the overlying normal blood vessel in the retina and choroid in the treated lesions were not destroyed, even after the application of the PDT laser at maximum energy. Probably because neither the dendrimer-porphyrin loaded micelles nor free dendrimer-porphyrins was taken up into the endothelial cells in normal blood vessels.¹¹¹ Dissociation of DP-loaded micelles was assumed to be gradual, and no

skin phototoxicity was observed which is in contrast to the results observed after the injection of Photofrin.

Another interesting study was reported by Jang et al.¹¹² They developed a PIC, which was formed via an electrostatic interaction of anionic dendrimer phthalocyanine (DPcZn) and PEG-*b*-PLL BCP (DPcZn/m) (Fig. 5(j)). DPcZn/m exhibited a strong Q band absorption around 650 nm, which is a better wavelength for deep tissue penetration compared to dendrimer-porphyrin with a relatively short wavelength absorption around 430 nm. The efficacy of PDT was greatly improved such that following 60 minutes irradiation *in vitro*, DPcZn/m exhibited almost 100 times higher phototoxicity compared with the free DPcZn.¹¹² Interestingly, DPcZn/m showed almost three to four fold decrease in oxygen consumption (indicating a decrease in quantum yield of singlet oxygen formation) and only four times higher cellular uptake when compared with free DPcZn.¹¹² Moreover, a subsequent study was performed to explore the underlying mechanism that led to enhanced phototoxicity by DPcZn/m. The study firstly demonstrated that DPcZn/m showed much higher phototoxicity compared to free DPcZn and consequently achieved significantly higher *in vitro* phototoxicity against A549 human lung adenocarcinoma cells, and better *in vivo* response in mice bearing A549 tumors compared with Photofrin (a clinically used PS). There were marked differences in the fluence-rate-dependence of phototoxicity between DPcZn/m and free DPcZn and considerable differences were observed in morphological changes of the cells during PDT. DPcZn/m induced rapid cell death accompanied by swelling and membrane blebbing, that are characteristic changes of oncosis, which is a type of cell death induced by hypoxia, inhibition of ATP production and increased plasma membrane permeability.¹¹³ On the other hand, free DPcZn induced different morphological features such as gradual shrinkage of the cells. Previous studies have suggested that the sub-cellular distribution of PS during irradiation as well as its redistribution to different organelles following irradiation, are among the key elements determining the PDT efficacy and type of response. For example, Li et al. have previously shown that exogenous PpIX located in the plasma membrane had a lower PDT effect than ALA-induced PpIX in the mitochondria.¹¹⁴ Savic et al. had also demonstrated that formulation in copolymer micelles altered the cellular distribution of 5-dodecanoylamino fluorescein and increased its uptake in PC12 cells.¹¹⁵ Similarly, PEG-attached dendrimers containing PpIX provided enhanced singlet oxygen generation as well as enhanced accumulation of the PS in the mitochondria of HeLa cells in comparison to free PpIX.¹¹⁶ In terms of cellular localization, both DPcZn/m and free DPcZn selectively accumulated in the endo-/lysosomes suggesting their cellular internalization through endocytosis. However, once the cells were photo-irradiated, the PS may have translocated from the endo-/lysosomes to the cytoplasm because DPcZn fluorescence became diffuse in both DPcZn and DPcZn/m PDT treated cells. This phenomenon is called photochemical internalization¹¹⁷ or relocalization which enables cytoplasmic delivery of cell-membrane-impermeable low-molecular-weight drugs and macromolecular compounds through light-induced photochemical disruption of endo-/lysosomal membranes (Fig. 6).^{118–120} Upon photo-irradiation, while DPcZn/m showed appreciable ROS production in the mitochondria, no mitochondrial ROS production was observed with free DPcZn/m. The experiments indicated that DPcZn/m may induce photo damage to the mitochondria resulting in exhaustion of ATP in the cells which would explain

the oncosis-like death pattern. It should also be noted that severe damage to the skin and liver was observed in Photofrin (8.1 $\mu\text{mol/kg}$) treated mice. However, DPcZn/m (4.2 $\mu\text{mol/kg}$) treated mice did not induce such damage under the tested conditions.

An exciting alternative to increase deep tissue penetration is through two-photon absorption (TPA) induced excitation, which is based on the absorption of two NIR photons. Two of these photons 'in the range of 750–1000 nm' provide same level of energy to excite electrons as a single photon of half the wavelength. TPA can also be combined with Förster resonance energy transfer (FRET), which is the non-radiative energy transfer from the donor to an acceptor.¹²¹ The main idea behind using FRET-based singlet oxygen generation is the chemical/physical incorporation of a donor having high absorption coefficient and also fluorescence quantum efficiency in close proximity to an acceptor PS within nanoscopic distance for effective energy transfer. As a result of activation of a donor via excitation with an appropriate wavelength, the donor transfers the energy (without and photon emission) to the acceptor, which is the PS in case of PDT. Following series of events as discussed earlier in this review, singlet oxygen generation is achieved.

Porphyrin PS have low TPA cross-sections which limits their use in TPA applications. Dichtel and colleagues suggested a new approach such that a dendritic array of eight donor TPA efficient chromophores were covalently attached to a central porphyrin acceptor.¹²² After TPA, the donors transferred their excited state energy to the porphyrin via FRET where intersystem crossing and singlet oxygen generation occur. Two-photon excitation of the donor chromophores at 780 nm resulted in a dramatic increase in porphyrin fluorescence relative to a porphyrin model compound.¹²² This new approach also enabled other donor chromophores or functional groups to be incorporated into future designs without interfering with the desirable properties of the central porphyrin acceptor.

The use of dendrimers for systemic delivery of PS over a longer period may have potential applications based on previous studies using fractionated PS dosing.^{123–125} Investigators reported better treatment efficacy and tumor selectivity using fractionated PS dosing when compared to single bolus dosing in a pancreatic tumor model. A limitation that still remains a challenge is the diversity of release mechanisms and the range of release kinetics. When the PS is only encapsulated in a dendrimer, the payload may be released prematurely before the macromolecules can reach the target site, whereas when the is conjugated in a PS-dendrimer, the release of PS relies primarily on destruction of the chemical linkage connecting the drug to the dendrimer periphery.⁹⁴ A well designed, customized multi-valent dendritic carrier that can support one or multiple drugs, a targeting moiety, a contrast agent to visualize delivery as well as a diagnostic sensor to detect the extent of the inflicted cell death. Therefore, this type of theranostic construct has the potential to overcome most of the challenges that PDT is facing today.

Porphysomes

Porphysomes are spherical nanovesicles formed from self-assembled porphyrin bilayers and their subunits consist of porphyrin-lipid conjugates which are generated by an acylation reaction between lysophosphatidylcholine and pyropheophorbide, a chlorophyll-derived porphyrin analogue.^{126, 127} Unlike conventional liposomal carriers where PS are inserted

into either the core or lipid-bilayer of the liposome, pyropheophorbide is directly conjugated to the phospholipid and the porphyrin bilayer contains a high porphyrin packing-density.¹²⁷ Porphysomes are biodegradable and were shown to induce minimal acute toxicity in mice upon intravenous injection.¹²⁶ In contrast with most other NP, which do not intrinsically absorb light in the NIR region, they demonstrate high absorption of NIR and generate tunable extinction coefficients. Moreover, they demonstrate structure-dependent fluorescence self-quenching that enables *in vivo* fluorescence imaging upon destruction of the porphyrin. Porphysomes possess unique photothermal as well as photoacoustic properties which enable non-invasive detection of circulating cancer cells in blood vessels as well as cancer cells in sentinel lymph nodes while maintaining the drug delivery capacity and biocompatibility of conventional liposomes.¹²⁸ Once porphysomes are taken up by cells via endocytosis, the structure of the porphysomes is destabilized and the fluorescence is greatly enhanced, which in turn serves as an indicator that the porphysomes have reached the target site when used in fluorescence imaging. Due to their liposome-like structure, their membrane has the potential to be modified through active targeting strategies used to functionalize conventional liposomes, such as conjugation of an antibody, peptide or transferrin.¹²⁷ For example, inclusion of 1 molar% folate-PEG-lipid enabled formation of folate-receptor-targeted porphysomes, and specific uptake by KB cells was demonstrated by confocal microscopy.¹²⁶

CONCLUSION

While PDT has been established for more than four decades, and is approved for a wide range of oncological and non-oncological indications, its clinical impact with respect to cancer therapy is still limited. For most clinicians, PDT does not offer strong enough advantages over other treatment alternatives, such as surgical resection, targeted radiation, or chemotherapy. While safe, systemically delivered PS agents with imaging and therapeutic capability can be used in conjunction with specific light delivery to target tissues, why is PDT still not the first treatment of choice ?. Limited light penetration is one reason that makes tumor treatment more challenging. This can be overcome by light delivery through optical fibers, even though this adds further complexity to the treatment. Patient discomfort due to skin photosensitization which may last up to weeks is another drawback, although new generation of PS have been developed to reduce this period of light avoidance.

The overall goal of PDT is to destroy the tumor tissue, while leaving healthy tissues undamaged. However, while tumors take up PS with some selectivity, some PS also accumulate in surrounding healthy tissues. Thus, another limiting factor of PDT is that light directed at the tumor site may damage adjacent healthy tissues. This prevents the use of higher PS amounts, which potentially leads to incomplete treatment responses. In order to solve this problem, many laboratories have been working on the development of new methods for site-specific delivery of PS such as encapsulation of PS into drug delivery vehicles.

The self-assembling methodology for preparing nanoconstructs provides a platform for the successful integration of diagnostics and therapy, which is vital for the future of customized treatments such as theranostics. Micelle-based drug delivery has created a plethora of

possible configurations that can allow encapsulation of drugs of various types, can allow targeting with better temporal and spatial resolution, and enable control release of the drugs. This has the potential to be a transformative approach, since if the PS activity is limited to the tumor, larger light doses and PS doses can be used to give better tumor destruction, while at the same time leaving adjacent healthy tissue undamaged. The wide variety of the possible BCP structures allows future researchers to find the best non-invasive ways to tailor nano-drug delivery vehicles by identifying the optimal structural configuration created by self-assembly. PDT based-activation incorporated with nanotechnology can further enhance effective drug-delivery while minimizing side-effects, and is expected to be clinically applicable in the near future.

Acknowledgments

We are grateful to Timur Zhiyentayev for his valuable input and discussions. Research in the Hamblin laboratory is supported by US NIH grant R01AI050875.

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ALA **Methyl-ALA** **Porphyrin** **Phthalocyanine** **Chlorin** **Bacteriochlorin**

Figure 1.
General structures of photosensitizers.

Diblock Copolymer

Multiblock Copolymer

Graft Copolymer

Figure 2.

Different basic types of block copolymers. The term ‘block’ denotes the linear architecture of the copolymer in which the end of one segment is covalently joined to the head of the other segment to give diblock or multiblock type copolymers. Graft copolymers resemble a comb with hydrophilic segments attached on the side of cationic segments. Reprinted with permission from [25] Y. Kakizawa and K. Kataoka, Block copolymer micelles for delivery of gene and related compounds. *Adv. Drug Deliv. Rev.* 54, 203 (2002). © 2002, Elsevier.

Figure 3.

Polyionic complexes. Block copolymers containing hydrophilic segments connected to segments of opposite charges will form a core in which the charged portions bind to each other via static charge interaction and the hydrophilic regions form the shell. The resulting tertiary structures can be thought of as micelles. Reprinted with permission from [25], Y. Kakizawa and K. Kataoka, Block copolymer micelles for delivery of gene and related compounds. *Adv. Drug Deliv. Rev.* 54, 203 (2002). © 2002, Elsevier.

Figure 4.

Structures of Photosensitizers: (a) meta-tetra(hydroxyphenyl)chlorin (m-THPC); (b) 5,10,15,20-tetrakis(meso-hydroxyphenyl) porphyrin (m-THPP); (c) chlorin(e6) (Ce6); (d) Protoporphyrin IX; (e) trimeric 3m ALA with amino-substituted core; (f) trimeric 3H ALA with nitro-substituted core; (g) 3 ALA with benzyl core.

Figure 5.

Structures of dendrimeric photosensitizers. (h) Nbis{*N*-(tris-5-aminolaevulinyloxymethylmethyl)propionamido}-propionamido}carbamidobenzene 18 trifluoroaceticacid (18m-ALA); (i) G3-poly(benzyl ether) dendrimer, composed of 32 negatively-charged carboxylate periphery groups having a zinc porphyrin in the center; (j) Anionic dendrimeric phthalocyanine having 32 methyl esters (DPcZn).

Figure 6.

Photochemical internalization. A nanoparticle is taken up by endocytosis into endo-/lysosomes. Following photoactivation it may escape into the cytoplasm and relocate to the mitochondria where the PS is more active. Reprinted with permission from [34], N. Nishiyama, et al., Design and development of dendrimer photosensitizer-incorporated polymeric micelles for enhanced photodynamic therapy. *Adv. Drug Deliv. Rev.* 61, 327 (2009). © 2009, Elsevier.