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Self-assembled liposomal nanoparticles in photodynamic therapy

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Abstract

Photodynamic therapy (PDT) employs the combination of non-toxic photosensitizers (PS) together with harmless visible light of the appropriate wavelength to produce reactive oxygen species that kill unwanted cells. Because many PS are hydrophobic molecules prone to aggregation, numerous drug delivery vehicles have been tested to solubilize these molecules, render them biocompatible and enhance the ease of administration after intravenous injection. The recent rise in nanotechnology has markedly expanded the range of these nanoparticulate delivery vehicles beyond the well-established liposomes and micelles. Self-assembled nanoparticles are formed by judicious choice of monomer building blocks that spontaneously form a well-oriented 3-dimensional structure that incorporates the PS when subjected to the appropriate conditions. This self-assembly process is governed by a subtle interplay of forces on the molecular level. This

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review will cover the state of the art in the preparation and use of self-assembled liposomal nanoparticles within the context of PDT.

Keywords

liposomes; nanoparticles; nanotechnology; photodynamic therapy; self-assembly

Introduction

Nanotechnology has become one of the great growth areas in 21st century science. As yet most of the commercial applications of nanotechnology have been in the materials sciences and nanoparticles are rapidly being incorporated into industrial and consumer products. The area of research that is producing most excitement, however, is probably that of biomedical research and the burgeoning field of nanomedicine. The nanometer scale of nanomedicines is considered to be ideal to interact with cells that have dimensions (microns) that allow them to efficiently interact with nanoparticles (10–200 nm). Many (if not most) drugs can be made significantly more effective if delivered using appropriate drug-delivery vehicles that allow them to efficiently reach their target in a form that both enables the drugs to be taken up by cells and minimizes off-target effects. More efficient and accurate delivery to their targets is expected to reduce the side effects of nanomedicines compared to conventional delivery. Self-assembly is an elegant, “bottom-up” approach to fabricating nanostructured materials. The alternative “top-down” approach to fabricating nanoparticles by milling and/or scanning microscope tips is technologically impractical due to lack of precise control and the large amount of time required. Therefore, nanoengineers manipulate the nanoparticles’ properties by various treatments so that they can simply “dump” the precursors into the mixture, whereby they automatically arrange themselves.

Photodynamic therapy (PDT)

PDT was discovered over one hundred years ago by the serendipitous observation that microorganisms stained by acridine orange were inactivated when exposed to light (1). It was not long afterwards, when PDT was used to treat skin cancer by topical application of a dye and subsequent illumination (2). The modern use of PDT to treat cancer arose in the 1960s because of the observation that injected porphyrins accumulated in tumors (3, 4). The mechanism of action has been determined to be the absorption of a photon that excites the electron in the highest occupied molecular orbital in the photosensitizer (PS) (5). The excited singlet state can undergo intersystem crossing to form a long-lived triplet state that can carry out photochemistry by undergoing an energy transfer or an electron transfer reaction with ambient ground state oxygen. The reactive oxygen species (ROS) formed (singlet oxygen and hydroxyl radicals) can oxidize biomolecules and subsequently kill undesirable cells such as cancer cells, microorganisms and neovascularization. The process is illustrated by the Jablonski diagram in Figure 1.

One of the main challenges to overcome in PDT is drug delivery to the target tissues. The poor water solubility of most efficient PS and their tendency to aggregate under physiological conditions are the key limitations, since the monomeric state is required to maintain their photophysical, chemical, and biological properties for efficient PDT effects (6, 7). Moreover, the accumulation and selective recognition of target tissues or cells is often sub-optimal. In order to improve PS delivery to the target tissue, nanomedicine offers alternative strategies. For example, certain nanoparticles have the ability to increase the solubility of hydrophobic drugs, and increase hydrophilicity and also have the proper size to accumulate in the tumor tissue via the enhanced permeability and retention effect (EPR) (8).

Selective drug delivery and accumulation may be improved through modification of the surface area of nanoparticles through selection of internalizing ligands for enhanced receptor-mediated nanoparticle uptake and the development of extracellular targeting ligands for vascular tissue accumulation of nanoparticles (9). However, attention also has to be given to the potential drawbacks of these systems such as lack of controlled release, prolonged tissue exposure, unknown long-term effects, stability and alteration of the photophysical properties of the PS (8, 9). In fact, many toxic effects of nanoparticles have been reported which range from increased inflammation, to lung tumor induction, impairment of cardiac function, higher levels of oxidative stress, platelet aggregation and many others (10, 11). Another limitation of using nanoparticles is that following administration, they are rapidly removed from the circulation by macrophages of the reticuloendothelial system (RES) and accumulate in the spleen and the liver (12). On the other hand, several solutions have been suggested to prolong the circulatory half-life of nanoparticles; such as using functionalized lipids in their construction where nanoparticles show longer circulation in the blood, demonstrate less reactivity towards serum proteins and are susceptible to RES uptake (8). The ideal PS delivery system should be nontoxic with no side effects, be biodegradable, small in size but have high loading capacity, minimum immunogenicity and should demonstrate controlled release characteristics. Furthermore, it should show prolonged circulation in the body following intravenous administration, have a minimal tendency to self-aggregate and should selectively accumulate in the target area with the required therapeutic concentration and with little or even no uptake by non-target cells (8). Recently, the focus on PS drug delivery has shifted toward developing nanoparticles through self-assembly or high-throughput processes to facilitate the development and screening of nanoparticles with these distinct properties and the subsequent scale-up of their manufacture (9). Parallel efforts have been undertaken to integrate additional functionality within therapeutic and imaging nanoparticles, including the ability to carry more than one payload, to respond to environmental triggers such as pH, temperature and light to activate PS release and to provide real-time feedback (9).

Self-assembly mechanisms

Self-assembly is a process in which a disordered system of pre-existing components form an organized structure as a result of certain specific, local interactions among the components themselves, without any external direction. When the constitutive components are molecules, the process is termed molecular self-assembly. It can be classified as either static or dynamic. In static self-assembly; the ordered state forms as a system approaches towards equilibrium, reducing its free energy whereas in dynamic self-assembly; patterns of pre-existing components organized by specific local interactions are not commonly described as self-assembled but are better described as self-organized (13).

Molecular self-assembly takes place without guidance or management from an outside source. There are two major types of molecular self-assembly, intramolecular self-assembly and intermolecular self-assembly. Intramolecular self-assembling molecules are often complex polymers with the ability to assemble from the random coil conformation into a well-defined stable structure (secondary and tertiary structure). Thus, molecular self-assembly is a key concept in supramolecular chemistry since assembly of the molecules is directed through noncovalent interactions (e.g., hydrogen bonding, metal coordination, hydrophobic forces, van der Waals forces, π - π interactions, and/or electrostatic as well as electromagnetic interactions). Common examples include the formation of micelles, vesicles, liquid crystal phases, and Langmuir monolayers by surfactant molecules (14).

The major features that make self-assembly a distinct process are the order, interaction and the building blocks (15). The self-assembled structure must have a higher order than the

isolated components, be it a particular task that the self-assembled entity may perform or the shape of the entity. The other important aspect of self-assembly is the key role of weak interactions. It is important to note how weak interactions hold a prominent place in materials science, and especially in biological systems, though they are often neglected compared to strong (i.e., covalent, etc.) interactions. For example, weak interactions determine the physical properties of liquids, the solubility of solids, and the organization of molecules in biological membranes. Finally, the building blocks are very important aspect in self-assembly. They span a wide range of nano- and mesoscopic structures, with different chemical compositions, shapes and functionalities. These nanoscale building blocks can in turn be synthesized through conventional chemical routes or by other self-assembly processes.

Researchers have used the self-assembled mechanisms to produce nanoparticles with applications in RNA nanotechnology (16), proteins (17), enzymes (18), silica mesoporous materials (19) and microfluidic particle crystals (20). Recently, Giacometti et al. (21) explained the self-assembly mechanism in colloids. They discussed a self-assembly mechanism from protein to patchy colloids following a simple theoretical model, the Kern-Frenkel model (22) describing a fluid of colloidal spherical particles with a pre-defined number and distribution of solvophobic and solvophilic regions on their surface. Enzymatic self-assembly is another concept and examples include esterase based self-assembly of nanofibers for regulating cell death, phosphatase based self-assembly of Taxol®-nanofibers for cancer therapy, hemolysin catalyzed self-assembly of nanofibers for immobilizing laminin to treat extra cellular matrix (ECM) diseases and application of enzyme-catalyzed or regulated formation of nanostructures for diagnosis and theranostics therapy (18).

Application of self-assembled nanoparticles in PDT is being examined by several investigators. For example, Li et al. formed novel heparin-based self-assembled nanoparticles containing heparin-poly(β -benzyl-L-aspartate) (HP) amphiphilic copolymer for effective delivery of a hydrophobic PS, pheophorbide a (Pheo) to SCC7 cancer cells, showing its potential as an effective delivery system for clinical PDT (23). Li et al. used Chlorin(e6) (Ce6) (Figure 2A) conjugated to chondroitin sulfate (CS) synthesized through the formation of an ester linkage between CS and Ce6 and evaluated these compounds as potential nanoscale drugs for PDT through self-assembly mechanism (24).

Another approach to self-assembled structures which has been particularly successful is the controlled self-assembly on surfaces. There have been a range of different molecular systems that self-assemble, forming ordered, monomolecular structures by the coordination of molecules to surfaces. These self-assembled monolayers (SAM) (25) are increasingly useful in various technologies and the mechanisms are reasonably well understood. Thus, SAM represent the kind of structure that uses molecular self-assembly to build structure and function on the nanometer scale. They are also the first of the self-assembled systems to move into technology transfer in nanotechnology. The application of self-assembly could play a key role in the area of PDT forming the theranostics molecular moiety with different functionalities assembling all together to perform multiple applications.

Liposomes

Liposomes are colloidal carriers that are used to transport and deliver therapeutic drugs such as PS through the body to the specific target by protecting the drug against degradation while preventing adverse side effects when PS is illuminated with appropriate wavelength. These liposomes are formed by self-closed spherical nanostructures with one or more concentric lipid bilayers (26) with an aqueous inner core (~50–150 nm in diameter) (27) and a lipophilic region between the polar head groups of one or more lipid bilayers of natural

and or synthetic lipid (28). The bilayers form unilamellar vesicles (100 nm–800 nm in size and formed by a single bilayer) or multilamellar vesicles (500–5000 nm in size and consisting of several concentric bilayers) (26, 27). The multilamellar vesicles have a stable encapsulating mechanism for the drug and exhibit a remarkably controlled rate of release compared to that of the unilamellar liposomes (29). Liposomes are highly biocompatible and biodegradable nanocarriers (especially as phospholipid vesicles) (27). These properties make liposomes powerful drug carrying systems (30). Drugs with widely varying lipophilicities can therefore be encapsulated in liposomes by localizing in one or more of the following domains (shown in Figure 3): (i) in the phospholipid bilayer (ii) in the aqueous core and (iii) in the bilayer interface (28). With their special capability to encapsulate large amount of drugs, liposomes are flexible enough to accommodate entities such as PS with varying physicochemical properties (31).

Conventional or unmodified liposomes have their own limitations, for instance in the case of tumor selectivity for the target PS. One of the main reasons for this limitation is due to the plasma half-life of the liposomes that is too short for efficient tumor uptake to occur by the enhanced permeability and retention (EPR) effect (32). This limitation can be overcome by using modified liposomes (especially those with long half-life and active targeting functionality) that have the capability to deliver more drugs. The development of surface modification chemistry of liposomes unfolds a plethora of possible modified structures and novel liposomes that can be subcategorized associating with their biocompatibility, transportability and therapeutic capability.

Liposomes can be broadly classified according to their composition, size (33) and delivery mechanism. With respect to their composition and mechanism of intracellular delivery, liposomes can be classified as following: (i) conventional liposomes; (ii) pH-sensitive liposomes; (iii) cationic liposomes; (iv) immunoliposomes; and (v) long-circulating liposomes. In terms of their size and lamella liposomes fall under three types (i) multilamellar vesicles (ii) large unilamellar vesicles, and (iii) small unilamellar vesicles. All of the above category of liposomes, depending on how they are being directed to the target (site-specific targeting) and drug release can be further classified according to the following: (i) actively targeted liposome (ii) passively targeted liposomes and (iii) triggered release liposomes.

There is an array of “somes” that fall under the category of liposome. However, this review solely concentrates on the liposomes that are being used for PDT applications. Jin and Zheng (31) reviewed how the composition, size, phospholipid properties, and liposome formulation are correlated to the delivery mechanism of PS. PDT is generally applied as a single modality for the treatment of a variety of malignant cells by its dominant mechanism of action of generating cytotoxic singlet oxygen locally, which causes the destruction of the malignant cells by photokilling (34). Since the lipid bilayer of liposomes can incorporate highly hydrophobic PS and prevent their aggregation, and also possess the capability to incorporate hydrophilic PS in the aqueous core, they are considered versatile nanocarriers for PDT applications.

Conventional liposomes for PDT

Conventional liposomes are composed of phospholipids but also cholesterol is often included as a constituent. Combining PDT with chemotherapy is a novel approach that exploits the vascular permeabilizing effects of low fluence rate/low fluence PDT to improve delivery of macromolecular drug carriers. For instance, a study by Snyder et al. combined liposomally encapsulated doxorubicin (Doxil) with PDT using 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide- α as a PS and demonstrated that combination therapy led to significant inhibition of tumor growth without increasing the local or systemic toxicity (35).

The combination of PDT and Doxil led to a highly significant potentiation in tumor control without local toxicity. In another PDT study the PS (Photofrin which is a complex mixture of hydrophobic dimers and oligomers primarily linked by ether bonds) was formulated in liposomes. This process significantly increased efficiency against a human glioma implanted in rat brain when compared to non-liposomal Photofrin (32).

Visudyne® (QLT Phototherapeutics, Vancouver, Canada) is one of the most successful examples for the use of liposomal delivery and was the first liposomal drug approved by the FDA in 2000 for the treatment of age related macular degeneration (36). It is a nonpegylated liposomal formulation that encapsulates benzoporphyrin derivative monoacid ring A (BPD-MA, Figure 2B). It is supplied as a freeze dried preparation composed of egg phosphatidyl glycerol and dimyristoyl phosphatidylcholine (BPD/EPG/DMPC; 1.05:3:5 w/w/w) (37). Visudyne is being widely used in ophthalmology as a PS in combination with trans-pupillary red laser for destroying neovascularity in the eye secondary to diseases such as wet age-related macular degeneration (38). Other than its use for age related macular degeneration, a combination of verteporfin PDT and immuno-suppression was proposed to be beneficial for treatment of subfoveal choroidal neovascularization (CNV) which can occur as a complication of inflammatory conditions (39–42). PDT with Verteporfin (Visudyne®) has also shown beneficial effects compared with no treatment in selected cases of CNV secondary to pathologic myopia (43). Lastly Gross et al. used a modified liposomal formulation (Verteporfin encapsulated in cationic liposomes) and achieved reduced PDT-associated retinal damage while demonstrating the same choroidal antineovascular efficacy as Visudyne alone (44).

A second successful application of liposomes in PDT drug-delivery was that of zinc phthalocyanine (ZnPC, see Figure 2C). ZnPC is highly insoluble and was formulated by Ciba-Geigy Pharmaceuticals. These liposomes are composed of palmitoyl-oleoyl-phosphatidylcholine and di-oleoyl phosphatidylserine (ZnPC/POPC/OOPS; 1:90:10 w/w/w) to form CGP55847 (45). Although CGP55847 was tested in clinical trials of PDT for squamous cell carcinomas of the upper aerodigestive tract (46) it never received regulatory approval.

Another PS tested for PDT is m-Tetrahydroxyphenyl chlorin (mTHPC, Figure 2D) (with the generic name 'Temoporfin' and the proprietary name 'Foscan®') (47– 50). Foslip® on the other hand, is a liposomal formulation of the PS mTHPC, consisting of mTHPC encapsulated in conventional liposomes and it is a clinically approved PS that is widely used in Europe for PDT of head and neck and other cancers (51). Moreover, it was demonstrated to improve wound healing and reduce scarring (52). de Visscher et al. suggested higher bioavailability for the liposomal formulations over Foscan®, due to the marked differences these three mTHPC formulations showed in their fluorescence kinetic profile (53). Vascular mTHPC fluorescence increased for Foslip® but decreased for Foscan®. Furthermore, Foslip® showed a higher tumor selectivity at all time points, while Foscan® failed to do so (53).

Long circulating-PEGylated or “Stealth” liposomes

One of the potential limitations of conventional liposomes is the short circulatory half-life. These conventional liposomes consist of naturally occurring phospholipids and cholesterol, which are crucial for membrane stability, fluidity and modulation of membrane-protein interactions (32). The reactivity of lipids with serum proteins induces opsonization, thus, rapid clearance of liposomes from the circulation by RES macrophages. However, it is essential that liposome systems remain in circulation long enough in order to accumulate the therapeutically cytotoxic concentration of drug within the tumor tissue.

The circulatory half-life of the liposomes can be significantly increased by using functionalized lipids to make sterically stabilized or “Stealth” liposomes. Stealth liposomes can evade interception by RES, thus resulting in substantial increase in circulation time in vivo and help maintain therapeutically effective blood concentration (54–56). Polyethylene glycol (PEG) (57, 58), a water-soluble polymer that exhibits protein resistance, low toxicity, non-immunogenicity and antigenicity and can be prepared synthetically with high purity and in large quantities, is most widely used polymeric steric stabilizer for clinical applications (26, 59). The relative half-life of pegylated liposomes is significantly increased (~45 h) compared to nonpegylated liposomes (few hours) (60). Although stealth liposomes have the required longer in vivo circulation time, one shortcoming of these systems is decreased interaction cells, suggesting diminished efficacy compared to conventional liposomes in transferring the PS to target cells (32, 61). However, further investigations are warranted to realize if this indeed is the case.

Long-circulating liposomes are being widely used in biomedical in vitro and in vivo studies (54) owing to the special protective polymer coating which allows a large number of advantages including 1) availability of surface-grafted polymer molecules to create an impermeable layer over the liposome surface, 2) demonstrate dose-independency, 3) non-saturability, 4) log-linear kinetics, 5) increased bioavailability and 6) slow down liposome recognition by opsonins and therefore subsequent clearance of liposomes (26, 62). PDT efficacy of long-circulating liposomes passively targeting a PS to the tumor tissue has been tested by using BPD-MA incorporated in glucuronide-modified liposomes (PGlcUA-liposomes) and results from this study showed that mice bearing a subcutaneous sarcoma exhibited a significant tumor regression and 80% cure rate upon intravenous injection and subsequent tumor illumination has been observed (63).

Fospeg[®] is another liposomal formulation of mTHPC which has been designed to improve the stability and prolong the half-life of mTHPC by coating with PEG – a synthetic hydrophilic polymer (64, 65), PEG incorporated forms a hydrated shell, which restricts the liposomes-proteins interaction in the plasma thereby reducing the uptake of liposomes by the reticuloendothelial system RES (31). The effects of density and thickness of PEG coating on in vitro cellular uptake, and dark- and phototoxicity of liposomal formulations (Fospeg) of the photodynamic agent m-THPC) was investigated (66). In the dark all Fospeg[®] formulations were less cytotoxic in comparison with m-THPC delivered by the standard solvent and cytotoxicity decreased by increasing PEGylation. It was observed that m-THPC delivered as Fospeg[®] was internalized by endocytosis and localized mainly in the Golgi apparatus and endoplasmic reticulum. The efficiency of cellular uptake of Fospeg[®] was reduced by 30%–40% compared to m-THPC in standard solution with less phototoxicity but without serious impairment of efficacy.

In an in vitro model the photodynamic characteristics of the PEGylated liposomal formulation of m-THPC (Fospeg[®]) using the human prostate cancer cell line LNCaP, was investigated (67). A comparison study between Foscan[®] and Fospeg[®] was carried out where Fospeg[®] showed higher intracellular uptake at different concentration and incubation time than Foscan. However, Fospeg[®] produced severe cytotoxicity than Foscan[®] at these concentrations and fluences. With Fospeg[®] the lowest concentration (0.22 μM) and fluence [180 mJ/cm^2] death of 50% of the cells 24 h post PDT was noted while with Foscan[®], an approximately 10 times higher concentration (1.8 μM) was required in order to achieve same level of cytotoxicity. In order to gain insight into the in vivo behavior of these formulations, a similar study by Reshetov et al. compared Foslip[®] and Fospeg[®] in terms of liposomal stability and redistribution of mTHPC in human serum (65). Inclusion of mTHPC into conventional (Foslip[®]) and PEGylated (Fospeg[®]) liposomes showed no effect on equilibrium serum protein binding compared with solvent-based mTHPC. It was found that

at short incubation times the redistribution of mTHPC from Foslip[®] and Fospeg[®] led to drug release and destruction of liposomes whereas at longer incubation times, the drug redistributed by release only (65). The release of mTHPC from PEGylated vesicles was delayed compared with conventional liposomes, but with minimal liposomal destruction (65). Thus, for long-circulation times the pharmacokinetic behavior of Fospeg[®] could be influenced by a combination of protein- and liposome-bound drug. In an in vivo study Bovis et al. compared Fospeg[®] with different degrees of pegylation (Fospeg 2% and 8%) to Foscan[®] in terms of biodistribution, bioavailability and circulation time which are crucial parameters in PDT PS delivery. Fospeg 2% and 8% treatment group enabled high amount of PS uptake in target tumor tissue (probably due to increased blood plasma circulation), improved selectivity, reduced required drug dose and drug light interval, reduced normal tissue damage, and less cutaneous photosensitivity with higher levels of efficacy as compared to Foscan[®] (68).

In a recent experiment the lethal effect and mechanism of cell death induction of Fospeg[®] on hepatocellular carcinoma was demonstrated with an aim to functionally analyze the impact of PDT on Huh-7 HCC cell line and on cell cycle protein expression (69). The results of the study indicated a statistically significant decline in viability and proliferation of Huh-7 cells following PDT, while maintaining Fospeg concentration and laser intensity below the individual thresholds of cytotoxicity.

Most of the above studies indicated that PEGylated liposomes (Fospeg[®]) are promising nanocarriers for the delivery of PS for PDT due to their reduced dark cytotoxicity and increased therapeutic efficacy. Overall Fospeg[®] proves to be a promising carrier for PDT.

Light-sensitive liposomes

Light-triggered release of liposomal drugs involves modified phospholipid molecules which undergo either photopolymerization (70–72), photosensitization by membrane anchored hydrophobic (73) or water soluble probes (72), photoisomerization (74), photooxidation (75) or the degradation of photocleavable lipids (75) upon irradiation. This modification is usually achieved by introducing photoreactive groups such as (1,2, bis(tricoso-10,12-dinyol)-sn-glycero-3-phosphocholine (DC_{8,9}PC) in the phospholipid molecule (76, 77). However, plasmalogen, a natural ether phospholipid, is an exception to this notion, since it reacts with ROS to generate lysolipid and in turn promotes drug release from the liposomes. Plasmalogen has been used in delivery of hydrophilic PS such as zinc phthalocyanine (ZnPc), tin octabutoxyphthalocyanine, or bacteriochlorophylls- α and accelerated transmembrane flux has been observed (75, 78). Another example of light-triggered release is use of liposomes containing photopolymerizable phospholipid bis-SorbPC in order to deliver a hydrophobic PS 1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (77). Upon irradiation with 550 nm visible light, oxygen radicals produced initiated the polymerization process of bis-SorbPC and the PS was released. Use of membrane-bound sensitizers and capacity of liposomes to photolyze under certain wavelengths suggests that light-triggered liposomes may be a potential strategy for PS delivery in PDT.

Enzyme-triggered liposomes

Enzymes are one of the most important tools in nanotechnology that possess exceptional biorecognition capabilities and outstanding catalytic properties. Enzyme-responsive nanoparticles are capable of exhibiting high specificity for the triggering stimulus. Considering that tumor cells often overexpress specific enzymes required for their migration, invasion or metastasis, enzyme triggered liposomes seem to be promising for cancer therapy (79). Phospholipase A₂ (sPLA₂) is considered particularly as a good candidate for enzyme-triggered drug release (80). sPLA₂ – IIA subtype in particular has

been identified in a variety of cancer types including prostate, colon, breast and pancreatic cancer (80). Matrix metalloproteases (MMPs) are also commonly investigated for their use in enzyme-triggered drug release since different subtypes are overexpressed in different diseases such as various types of cancer, rheumatoid arthritis and autoimmune blistering disorders of the skin (80). However, utilization of MMPs for tumor-specific drug release is more challenging than PLA₂ strategy since it requires synthesis of specialized lipopeptides that are substrates for MMP activation and these lipopeptides need to be incorporated into the liposome membrane. In order to accomplish this, Sarkar et al. incorporated sequence-specific collagen-mimetic triple helical peptides on the liposomal surface and achieved specific recognition, subsequent cleavage and drug release by MMP-9 only, without any content release by other proteolytic enzymes such as trypsin (81). For further enhancing specific tumor accumulation of PS in PDT, targeting of over-expressed receptors that induce intracellular internalization combined with enzyme-triggered release seems to be a promising strategy.

Fusogenic liposomes

Fusogenic viral proteins such as glycoprotein-H of the herpes simplex virus are usually coupled to the surface of virosomes and liposomes when non-internalising receptors are targeted so that the content can be delivered to the cytosol by membrane fusion between the liposomes and the target cells (32, 82). Tu and Kim demonstrated that when virosomes were coupled to glycoprotein H, this coupling resulted in improved cellular internalization and endosomal release of DNA from cationic liposomes (83). Based on the ability of Sendai virus to fuse readily with almost all types of mammalian cells, coating liposomes with Sendai virus coat-proteins became a commonly used strategy. However, since viral proteins define which cells will be targeted, in order to be able to achieve a tumor-selective targeting, it is necessary that these fusogenic proteins are shielded by a polymer coat and tumortropics should be coupled at the distal ends of these polymers (84–86). On the other hand, fusogenic lipids such as dioleoylphosphatidylethanolamine, through forming a columnar inverted hexagonal lipid-crystalline structure, improve the membrane fusion of the liposome and enhance the liposome's ability for drug release (82). It is commonly included in the formulation of pH-sensitive liposomes where acidic environment of endosomes increases membrane fusion and triggers the release of encapsulated drugs (87). To our knowledge, yet no experiments have been performed using fusogenic liposomes for PS release.

pH-Sensitive liposomes

Pathological areas such as tumors, infarcts and areas of inflammation usually have lower interstitial pH when compared to healthy ones (88). Moreover, during the course of endocytosis, pH decreases from the cytoplasm to the endosomes and then to the lysosomes. Based on these phenomenon, pH-sensitive liposomes have been developed for drug delivery. This strategy facilitates the delivery of highly hydrophilic molecules including PS into the cytoplasm (89). Liposomes are usually composed of phosphatidylethanolamine and amphiphiles which destabilize under acidic conditions. In order to generate pH-sensitive liposomes, pH-sensitive molecules that are charged at neutral pH but uncharged at acidic pH have been incorporated to liposome formulations. When used in PDT, lipid-layer of liposomes becomes unstable in this acidic environment, liposome membrane permeability increases and subsequently the PS are released (90, 91). Incorporation of PEG-conjugated lipids into pH-sensitive liposomes such as polylactic acid (PLA) may be beneficial for prolonging the circulation times and antibodies or ligands to cell surface receptors may be coupled to pH-sensitive liposomes for improved target delivery.

Thermo-sensitive liposomes

Thermo-sensitive liposomes can be made by grafting of certain polymers, which display a lower critical solution temperature (LCST) slightly above their traditional counterparts. These polymers dissolve below LCST and precipitate but when the temperature is raised above the LCST liposomal membrane breakdown takes place enabling drug release (88). Liposomes can be designed in such a way that they can undergo phase transition on the application of heat, which renders them more permeability and consequently releases their payload when compared to their traditional counterparts. When these thermosensitive liposomes are enhanced with an external physical energy, such as infrared laser or microwave, a certain degree of improved targeting is achieved. Using such a technique thermosensitive liposomes have demonstrated the ability to improve the delivery of anti-cancer agents, such as methotrexate, cisplatin and doxorubicin to the tumor site (92).

As of today use of thermosensitive in PDT has not been investigated widely. One study by Rijcken et al. tested the loading and stability of thermosensitive biodegradable mPEG-*b*-p(HPMAM-Lac₂) micelles containing PS (Si(sol)₂Pc) (93). The authors concluded that controlled release, stability of highloaded micelles in presence of serum, and high phototoxicity of the encapsulated PS suggest potential for further investigation in in vivo studies (93).

Targeted liposomes

Targeting strategy is mainly based on targeting overexpressed or uniquely expressed molecules that are on the cancer cells through coupling the nanoparticle with a ligand which is capable of recognizing and binding to cells of interest. For targeting, long-circulating liposomes are preferred over the conventional ones in order to prolong interaction time between the target and targeted liposome (32). The objective of targeting is to minimize undesired side-effects caused by the accumulation of non-specific PS. This can be overcome by selective target-binding and cellular internalization of the liposome-bound PS during photodynamic process. The binding of the molecule can be done covalently either directly to a hydrophobic anchor (covalently conjugated into the liposomal bilayer) or by attaching a spacer molecule as a bridge (hydrophilic moiety) (26).

Below we discuss two types of targeting; 1) Folate targeted liposomes and 2) Antibody and ligand mediated liposome targeting.

Folate targeted liposomes

Folic acid is an essential vitamin utilized by the cell during proliferation. Folate and its conjugates have extremely high affinity for the folate receptor (FR) and are internalized through receptor-mediated endocytosis (94). Folate targeted drug delivery has emerged as an alternative therapy for the treatment of many cancers. Due to its small molecular size and high binding affinity for cell surface folate receptors (FR), folate conjugates are able to deliver various molecular complexes to pathologic cells without causing harm to normal tissues (94). The overexpression of folate receptor on a variety of epithelial cancer cells including cancers of ovary, lung, kidney, breast, brain and colon has been reported, however further studies are necessary (95, 96). The interaction between the ligand and receptor affects the rate of cellular internalization that in turn influences the accumulation of liposomal drugs in the cells (97). Folate has been reported to have fast internalization and intracellular recycling rate that in turn speeds up the delivery rate of PS into the cells (98). Lee and Low conjugated folic acid to liposomes via a PEG spacer and discovered that FR-mediated endocytosis enhanced the internalization of folate-conjugated liposomes at 37°C (99). During the endocytosis process, liposomes conjugated with folate ligands were shown to traffic to the endosomes and then lysosomes and average pH of endosomes along the

trafficking pathway was reported to be 5.0 (100, 101). This phenomenon can be exploited for the pH-triggered drug release. Rui et al. demonstrated that folate-dipalmitoylcholine (1,2-di-O-(Z-1'-hexadecenyl)-sn-glycero-3-phosphocholine; DPPLC) liposomes greatly enhanced the potency of water-soluble antitumor agents via a selective folate-mediated uptake and acid-catalyzed endosomal escape mechanism (102). Based on this strategy Qualls and Thompson studied delivery of aluminum phthalocyanine tetrasulfonate (AlPcS4, Figure 2E) a water-soluble PS with [AlPcS4/DPPLC:folate liposomes] (91). At the end of the study, use of lower bulk [AlPcS4] concentrations, rapid plasma clearance of free [AlPcS4], and better phototoxic responses, due to higher intracellular [AlPcS4] concentrations combined with reduced collateral photodamage arising from misguided sensitizer accumulation were suggested as the potential advantages of this approach (91). Incorporation of a model PS (ZnTPP) into a folate-targeted liposomal formulation has been shown to lead to an uptake by HeLa cells (folate receptor positive cells) that is 2-fold higher than the non-targeted formulation which in turn resulted in improved photocytotoxicity (103). However, the same study also demonstrated that both folate-targeted and non-targeted liposomes localize in acidic lysosomes, which confirms that the non-specific adsorptive pathway is also involved (103).

Antibody and ligand mediated liposome targeting

Immunoliposomes are liposomes that carry antibodies attached to their surfaces. They have the ability to accumulate within a specific area of the body where an attached antibody recognizes and binds to its antigen (104). Immunoglobulins (Ig), IgG class in specific, and their fragments are the most frequently used targeting moieties for liposomes due to their ability to be attached to liposomes, without affecting liposomal integrity nor the antibody properties (104). Mir and colleagues recently developed a photo-immuno-conjugate-associating-liposome (PICAL) in which benzoporphyrin derivative monoacid A (BPD) and the Cetuximab antibody for epidermal growth factor receptor (EGFR) were incorporated into a stable Preformed Plain Liposome (105). Results have demonstrated that in addition to selective binding of Cetuximab to ovarian tumor cells and inhibition of EGFR signaling, a stable optical behavior and a higher fluorescence quantum yield have been achieved when compared to free-BPD. Another interesting study by a group of investigators aimed selective thrombosis of irregular tumor blood vessels through an antibody-PS conjugate (106). Human antibody L19, a marker of angiogenesis, is specific to the EDB domain of fibronectin and a chemical conjugate of the L19 antibody with the PS bis(triethanolamine) Sn(IV) chlorin (e6) has been used in order to arrest tumor growth in mice with subcutaneous tumors (106). Results were highly suggestive of potential use of antibody-PS conjugates for the therapy of superficial tumors (106). While PDT with the chemical conjugate of the L19 antibody with bis(triethanolamine)Sn(IV) chlorin (e6), by contrast, a PS conjugate obtained with an antibody of identical pharmacokinetic properties but irrelevant specificity did not show any significant therapeutic effect.

Transferrin receptors (TfR) are overexpressed on the surface of many tumor cells, hence antibodies against transferrin receptors, as well as transferrin itself, are also commonly used ligands for liposomal targeting to tumor cells (104). Derycke and De Witte. investigated use of transferrin-conjugated PEG-liposomes in order to improve the specificity of PS hypericin for HeLa tumor cells which overexpress transferrin receptors on their surface (107). However results were not encouraging. Transferrin-conjugated PEG-liposomes were less effective in targeting hypericin to tumor cells which was attributed to the amount of hypericin leaking out of the PEG-liposomes (107). On the contrary, a similar study by Gijssens et al. demonstrated enhanced PDT activity of the PS aluminum phthalocyanine tetrasulfonate when encapsulated into transferrin conjugated PEG-liposomes (108).

Peptides that target G protein-coupled receptors, cell penetrating peptides and RGD peptides, are other commonly used tumor-specific ligands in targeted liposomal drug delivery. Ala-Pro-Arg-Pro-Gly (APRPG) pentapeptide, a peptide specific to angiogenic endothelial cells, have been used by Ichikawa et al. to test the possible improvement in bioavailability of (PEG)-modified liposomes encapsulating BPD-MA in antiangiogenic PDT. Strongly suppressed tumor growth following laser irradiation at 3 h after injection, as well as vasculature damage in the dorsal air sac angiogenesis model have been reported (109).

Conclusion

Nanotechnology and its application in nanomedicine will continue to feature strongly in biomedical research. Drug-delivery technologies are finally making it out of the laboratory and into clinical practice. One of the problems that is being faced by the pharmaceutical industry is how medicines formulated in self-assembled nanoparticles can be produced under good manufacturing practice conditions, can have a satisfactory shelf life under feasible storage conditions, and can be reconstituted if necessary before being administered to the patient. The example of Visudyne[®] which is supplied as a freeze-dried liposomal preparation that could be simply dissolved in sterile 5% dextrose before IV injection shows that this challenge can be satisfactorily solved. The composition of many self-assembled nanoparticles being formed from naturally occurring lipids and biologically compatible polymers such as PGLA and PEG suggest that nanotoxicology considerations will not turn out to be deal-breaking as may be the case with other nanocarriers, for example single walled carbon nanotubes.

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Biographies

Magesh Sadasivam received his Dual Masters degree in Nanoscience by Research and Nanotechnology from Amity Institute of Nanotechnology, India. He worked as a graduate research trainee at Wellman Center for Photomedicine exploring various nanomaterials as effective photosensitizers for photodynamic killing of various drug resistant bacteria. His research interest lies in combining his knowledge in Computer Science Engineering and Nanotechnology for medical applications. His current work involves the design and development of microfluidic devices powered by cell phones for Point-of-Care diagnostic applications.

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Shanmugamurthy Lakshmanan, PhD received his two masters (MSc and MPhil) in physics from Bharathiar University, India. He further received a masters (MS) in physics from Stevens Institute of Technology and a PhD, from a joint program from New Jersey Institute of Technology and Rutgers–the State University of New Jersey. He is currently a Research Scientist at Dr. Hamblin's group at Wellman Center for Photomedicine. He has experience in fabricating nanoscale devices using a novel technology called “nanoscopic lens” for medical applications. His current interest is to incorporate nanoscopic lens techniques with PDT by identifying novel photosensitizers.

Rakkiyappan Chandran has a Masters degree, specialized in Bio-Nanotechnology. In 2010 his work involved in novel synthesis of silver nanoparticles for antibacterial application and has filed 3 patents for the same. He later was involved in DRDO (Defence Research Development Organisation, India) project in developing a cream formulation for controlled release. From March 2012, he had the opportunity to work with Dr. Hamblin at Wellman Center for Photomedicine, MGH. During this period he explored his interest in Theranostics approach of treating various diseases and his research involves photodynamic therapy for antimicrobial applications with various photosensitizers, which includes functionalized cationic fullerenes.

Ying-Ying Huang, MD received her MD in 2004 and MMed (Dermatology) degrees from China. She started as a post doctoral fellow at Wellman Center for Photomedicine in Dr. Hamblin's group. After 4 years of research experience she is now an Instructor at Harvard Medical School. Her research interests are in photodynamic therapy (PDT) for infections, cancer and mechanism of low-level-light therapy (LLLT). She has published 39 peer-reviewed articles and 15 conference proceedings and book chapters.

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Michael R Hamblin, PhD is a Principal Investigator at the Wellman Center for Photomedicine at Massachusetts General Hospital, an Associate Professor of Dermatology at Harvard Medical School and is a member of the affiliated faculty of the Harvard-MIT Division of Health Science and Technology. His research interests lie in the areas of photodynamic therapy for infections and cancer, and in low-level light therapy for wound healing, arthritis, traumatic brain injury and hair regrowth. He has published 218 peer-reviewed articles, over 150 conference proceedings, book chapters, has edited 3 major textbooks, and holds 8 patents.

Figure 1. Jablonski diagram

Initial absorption of a photon by the ground state of the PS gives rise to the short-lived excited singlet state. This state can lose energy by fluorescence, internal conversion to heat, or by intersystem crossing to the long-lived triplet state. PS triplet states are efficiently quenched by energy transfer to molecular oxygen (a triplet state) to give singlet oxygen (Type 2) or by electron transfer to oxygen or to biomolecules to give superoxide and hydroxyl radical (Type 1).

Figure 2. Representative chemical structures of PS that have been used in combination with nanoparticles

(A) Chlorin(e6) (ce6), (B) Benzoporphyrin derivative (BPD), (C) Zinc phthalocyanine (ZnPC), (D) m-Tetra(hydroxyphenyl)chlorin (mTHPC), (E) Aluminum phthalocyanine tetrasulfonate (AlPcS4).

Figure 3. PS encapsulated in liposomes by localizing in different domains

Water-soluble molecules or solid molecules can be loaded in the internal aqueous (hydrophilic core); amphiphilic molecules orient into liposomal bilayer (hydrophobic core); transmembrane proteins span the lipid bilayer with sites exposed to the aqueous phase.