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Liposomal delivery of photosensitising agents

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Photodynamic therapy is a clinically approved treatment for cancer and noncancer diseases. This modality utilises light-activatable chemicals (photosensitising agents) to capture photons and use light energy for the production of cytotoxic reactive molecular species. Most photosensitisers that are in use clinically or in preclinical development are hydrophobic and tend to aggregate in the aqueous environment, which limits their delivery and photosensitising efficiency. Liposomal delivery of photosensitisers will often overcome or decrease these problems. In addition, as with chemotherapeutic agents, liposomal formulations of photosensitising agents may help to achieve better selectivity for tumour tissue compared with normal tissue. Over the past years, liposomal photosensitisers have emerged as therapeutic agents in many experimental studies, and have obtained approval for clinical applications. Recent progress in liposomal technology further opens up the possibility of generating more selectively targeted photosensitisers encapsulated in liposomes. This review will cover progress in the use of liposomal photosensitisers, summarise current liposomal formulations, and project future directions for the liposomal delivery of photosensitising agents.

Keywords: drug delivery, liposomes, pharmacodynamics, pharmacokinetics, photodynamic therapy, photosensitisers, tumour targeting

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1. Introduction

Liposomes are vesicles composed of one or more concentric phospholipid bilayer(s) [1]. The use of lipids as the composition materials makes liposomes biocompatible. Similar to any bilayer membrane structure, liposomes have two compartments, an aqueous core and a lipophilic space between the lipid bilayer. This offers the flexibility to encapsulate both hydrophilic and hydrophobic drugs. The macromolecular size (typically 60 – 120 nm) enables liposomes to have a high loading capacity of therapeutic agents. These properties make liposomes an efficient and powerful carrier for the delivery of different types of drugs [1]. Recent progress in liposomal technology and molecular biology further allow liposomes to have targeting power to achieve selective delivery to specific biological targets [2]. Liposomal formulation is commonly used for the preparation of photosensitising agents in photodynamic therapy (PDT), and liposomal photosensitisers have been successfully used in both experimental studies and the clinical practice of PDT. An excellent review is available on current liposomal technology and its relevance to photosensitiser delivery and PDT effect [3]. The current status of liposomal formulation as a delivery system for various types of photosensitising agents will be the focus of this review.

2. Photodynamic therapy

PDT is an established modality for the management of both cancer and noncancer diseases [4,5]. This therapy involves the administration of a photosensitising agent,

either systemically or topically, followed by light activation with an appropriate wavelength matching the absorption of the photosensitiser [5,6]. As light with a longer wavelength has a deeper tissue penetration, light in the red or near-infrared region is commonly used in PDT. Upon activation by light, the photosensitising agent, localised in the target cell/tissue, reacts with molecular oxygen and generates cytotoxic reactive oxygen species, leading to oxidative damage to the target cell/tissue. Since its inception as a cancer therapy ~ 25 years ago, PDT has gained acceptance as a treatment for both cancer and noncancer diseases. Several photosensitisers have received regulatory approval around the world [4-6]. For instance, PDT with Photofrin® (Axcan Pharma, Inc.) is approved in the US and Europe for early- and late-stage lung cancer, Barrett's oesophagus and late-stage oesophageal cancer. The photosensitiser Foscan® (mTHPC) marketed by Biolitec Pharma, is approved in Europe for head and neck cancer treatment, verteporfin (Visudyne®, Novartis Pharmaceuticals) is approved in the US and Europe for the treatment of age-related macular degeneration (AMD), 5-aminolevulinic acid (ALA) is approved for the treatment of actinic keratosis (US and Europe) and its methyl ester, Metvix® (PhotoCure), for basal cell carcinoma (Europe).

For any cancer treatment modality, selective killing of tumour cells is the most important criterion for successful clinical application. The selectivity of PDT in tumour cell killing is achieved through two factors, the first of which is the selective delivery of light [4]. Existing fibre optical systems and various types of endoscopy make it possible to accurately deliver light confined to almost any organ of the body. Second, some photosensitisers can preferentially accumulate in the tumour tissue because of the abnormalities of tumour tissues, such as leaky blood vessels and poor lymphatic drainage. However, almost all existing photosensitisers do not display a high enough affinity towards tumour tissue. As a result, some degree of normal tissue damage is commonly observed following PDT treatments, although it has been documented that the healing of normal tissue after PDT at certain anatomic sites is excellent [4]. Solubility is another challenge facing currently available photosensitising agents. The hydrophobic nature of most photosensitisers makes intravenous drug administration a difficult task. To overcome the intrinsic low tumour selectivity and water solubility exhibited by most photosensitising agents, various drug delivery systems have been developed to modify the physicochemical, pharmacokinetic and biological properties of photosensitisers [7,8].

3. Photosensitising agents

3.1 Photophysical and photochemical properties

Photosensitising agents in PDT are compounds that are able to absorb light of a specific wavelength and transform the energy into the production of reactive oxygen species, mainly singlet oxygen (quantum yield ~ 0.3 – 0.6) [9]. These compounds generally have polypyrrolic structures in order to efficiently capture photons in the visible spectrum. Upon absorption of photons, photosensitising molecules are excited to a short-lived excited singlet state (< 100 ns) [10]. Molecules in this excited state can either decay back to ground state by emitting fluorescence or transfer to a longer-living excited triplet state (> 500 ns) via intersystem crossing. Only photosensitisers of the excited triplet states have long enough lifetimes to react with other molecules, leading to the generation of reactive oxygen species. Thus, a high quantum yield of the excited triplet state is necessary for any efficient photosensitiser. Photosensitiser molecules in the triplet state can either react with other nearby molecules by electron and hydrogen transfer with the generation of various reactive radicals (type I reaction), or more commonly, transfer energy to oxygen molecules, leading to the production of singlet oxygen (type II reaction).

3.2 First- versus second-generation photosensitisers

The terms first and second generation have been used with reference to photosensitisers in the PDT literature without clear definitions, thus giving rise to subjective interpretations. Nevertheless, an approximate generalisation may be that haematoporphyrin/Photofrin/haematoporphyrin derivative (HPD) and the photosensitisers preceding it are referred to as first generation, whereas those following the development of Photofrin are termed second generation. A frequent association with the so-called second-generation photosensitisers is that they have one absorption maximum further in the red than Photofrin (> 630 nm), higher extinction coefficients of absorption in the red, and are cleared more rapidly from the body. Acridine orange was one of the first photosensitising compounds reported by Raab ~ 100 years ago [11]. The first medical application of a photosensitiser was made by von Tappeiner and Jesionek, who used eosin and white light to treat skin tumours [12]. Porphyrins were introduced into medicine in the early twentieth century, with Policard being the first to report the red fluorescence of haematoporphyrin in an experimental rat sarcoma under ultraviolet light in 1924 [13]. However, clinical application of photosensitising agents did not begin until the 1960s when the HPD, a mixture of a large number of porphyrins that is obtained when haematoporphyrin is treated with strong acid, was prepared by Schwartz [14] and used by Lipson to diagnose human tumours [15]. After several encouraging animal experiment reports from different groups [16-18], Dougherty pioneered the clinical application of PDT by initiating the very first PDT clinical trial with HPD for malignant tumours [19]. The initial success of HPD in patient treatment stimulated intensive research into this photosensitiser and the mechanism of PDT. Since then, thousands of patients have been treated with PDT using HPD. Today, Photofrin, a partially purified preparation of HPD, has been approved in a number of countries for the treatment of cancer or precancer diseases [6], as described in Section 2. The history of using photosensitising agents for disease detection and therapy has been documented by Ackroyd *et al.* [20]. As more patients are treated with Photofrin, the problems associated with this first-generation photosensitiser become apparent [21]. As a chemical entity,

Photofrin is a mixture, which makes its manufacture and quality control difficult. From a photophysical aspect, Photofrin has weak absorption at a relatively short working wavelength (630 nm), which limits the effective treating area. In terms of drug delivery, Photofrin has low water solubility and an unsatisfactory tumour-seeking ability, leading to relatively poor tumour selectivity. Finally, with regards to its pharmacokinetic properties, Photofrin tends to linger in circulation and tissue for a long period of time, which translates to prolonged skin photosensitivity. Experience with this first-generation photosensitiser makes it apparent that overcoming these problems will lead to a good photosensitiser. The quest for an optimised photosensitising agent began with modifying the porphyrin structure. A number of photosensitising agents have been developed and were recently reviewed by Detty *et al.* [21]. These second-generation photosensitisers are all either pure chemicals or a mixture of a few well-characterised components, and show significant improvements over Photofrin in photophysical and pharmacokinetic properties; for example, strong absorption in the range of 650 – 800 nm where tissue penetration is optimal and relatively fast tissue clearance. However, many of these second-generation photosensitisers are still highly hydrophobic and display poor tumour selectivity, which points to the need for drug delivery systems that address these problems.

3.3 Delivery of photosensitising agents

Various drug delivery systems are used to formulate photosensitising agents, and current state-of-the-art systems have been reviewed by Konan *et al.* [7]. As discussed above, two primary purposes are associated with the application of drug delivery systems for photosensitising agents. One is to formulate them in preparations suitable for intravenous administration and the other is to increase tumour selectivity. It should be pointed out that although drug hydrophobicity often represents a delivery problem, it is, however, necessary for drug penetration through the cell membrane, and highly hydrophilic drugs generally have poor cellular uptake [22]. In PDT, common approaches used for the formulation of photosensitisers are the encapsulation of photosensitising agents in colloidal carriers, such as oil-based dispersions [23-26], micelle systems [27], liposomes [3] and biodegradable nanoparticles [28-31], and conjugation of the photosensitiser with hydrophilic polymers such as polyethylene glycol (PEG) [32-34] and polysine [35-37]. To enhance selective delivery of photosensitising agents, a number of targeting moieties have been conjugated to drug carriers or photosensitisers. These targeting ligands include lipoproteins [38], antibodies or antibody fragments [39], growth factors [40,41], transferrin [42-44], glucose transporters [45] and folate [46].

4. Overview of liposomal formulations

4.1 Classification and composition of liposomes

Depending on the presence of membrane coating with hydrophilic moieties to maintain colloidal stability, liposomes are generally classified into conventional liposomes (CLs) and sterically stabilised liposomes (SSLs) [47]. These two types of liposomes have unilamellar or multilamellar lipid membrane structures that commonly consist of phosphatidylcholine and cholesterol (Chol). For SSLs, a concentration of 4 – 6 mol% *N*-PEG-distearoylphosphatidylethanolamine is typically incorporated in the formulation. Membrane coating with hydrophilic polymers (e.g., PEG) or glycolipids (e.g., ganglioside GM_1) limits binding of serum opsonins to liposomes and prevents interaction with phagocytic cells in the circulation and the reticuloendothelial system. Therefore, SSLs generally have a longer circulation time than conventional liposomes; because of this, SSLs are also called long-circulating liposomes. Liposomes can also be divided into passively or actively targeted liposomes, depending on whether they have a specific targeting moiety conjugated on the membrane.

4.2 Accumulation of liposomes in tumour tissue

Similar to other macromolecules, the accumulation of liposomes in the tumour tissue is largely due to leaky tumour blood vessels and the impaired lymphatic systems exhibited by most tumour tissues [48]. Leaky blood vessels allow more liposomes to extravasate across the vasculature, and impairment of the lymphatic system leads to a prolonged retention of liposomes in the tumour interstitial area. This enhanced permeability and retention effect is the primary mechanism responsible for the selective tumour accumulation of both passively and actively targeted liposomes [49]. For the passively targeted liposomes, this is probably the only mechanism leading to selective tumour distribution, whereas in the case of actively targeted liposomes, the targeting moiety on the liposomal membrane further enhances selective targeting toward cells with specific biological markers.

4.3 Stability and bioavailability of liposomes

Any liposomal formulation needs to balance the liposomal stability in the circulation with drug availability/release once it arrives at the target tissue [47]. Under ideal circumstances, therapeutic agents are stably retained in a liposomal carrier and selectively released in the target tissue. Unfortunately, existing liposomal technology is still unable to selectively release liposomal drugs in the target tissue, and factors such as drug physicochemical properties, liposomal structures and tissue environments can all affect liposomal stability and the release of therapeutic agents from liposomes. Highly hydrophilic drugs can be stably carried within the liposomal aqueous compartment while in circulation, but low membrane penetration can limit the release of these drugs to the target tissue. Highly hydrophobic drugs are likely to associate with the lipid components of liposomes and redistribute to plasma proteins before reaching the target tissue. Amphipathic drugs have been shown to be the most suitable for liposomal formulation. Liposomal structure can also significantly affect liposomal stability and drug release. The presence of Chol and saturated phospholipids increases the rigidity of liposomes but reduces drug release, whereas liposomes containing more fluid lipid components

(e.g., egg PC) can easily break up and release the drug during circulation [47].

5. Liposomal photosensitising agents

5.1 Liposomal porphyrin photosensitisers

This category of liposomal photosensitisers includes HPD, Photofrin (partially purified form of HPD) and purified porphyrins such as tetraphenylporphyrins, tetramethyl porphyrins, tetrapyridyl porphyrins and protoporphyrin IX (PPIX).

HPD is the first photosensitiser encapsulated in liposomes to solve low solubility and aggregation issues [50]. Incubation with equivalent concentrations of either HPD in aqueous solution or HPD and its dimethylester bound to unilamellar liposomes in HeLa cells showed that liposomal porphyrins were able to bind to cells at a higher rate and in a considerably larger amount [51]. The release of cell-bound porphyrins into the cell culture medium was remarkably reduced and slowed using liposome-bound porphyrins. As a result, a dramatic increase in PDT efficiency was obtained by liposomal HPD. In the AJ-CR inbred albino female mouse implanted with C-1300 neuroblastoma, liposome delivery of HPD displayed favourable uptake in the tumour tissue [52]. In mice bearing MS-2 fibrosarcomas, Jori found that tumour uptake of HPD was higher for the liposomal delivery than for the aqueous delivery [53]. Jiang *et al.* encapsulated Photofrin in a unilaminar dipalmitoylphosphatidylcholine (DPPC) liposome and compared tumour drug uptake and response with PDT with Photofrin delivered in dextrose solution. It was found that liposomal Photofrin (injected intraperitoneally) led to enhanced tumour drug uptake and caused more tumour damage than Photofrin in dextrose, in both 9L rat gliosarcoma [54] and U87 human glioma xenograft in athymic nude rats [55]. Damage to normal brain tissue was, however, comparable between the two delivery systems. Similar results were obtained in a human gastric cancer xenograft using Photofrin entrapped in multilaminar liposomes composed of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol and Chol [56]. These results indicate that liposome formulation can enhance PDT efficacy by increasing photosensitiser tumour concentration.

Liposomal formulation has also been used to prepare purified porphyrins, including tetramethyl porphyrin [57], tetraphenyl porphyrin (TPP) [58,59] and dimeric porphyrin [60,61]. Formulation of these hydrophobic photosensitisers in liposomes not only enables them to be delivered by common administration routes, but also maintains photosensitisers in the monomeric state for the efficient production of singlet oxygen. Because of the hydrophobic nature of many porphyrin photosensitisers, certain structural features must be met in order to prepare stable and applicable liposomal formulations. By studying liposomes with metallated and nonmetallated TPPs and tetrapyridyl porphyrins using different lipid/porphyrin ratios, Postigo *et al.* recently demonstrated that the incorporation of porphyrins into liposomes can be related to their ability to form aggregates in a watery media. They found that ZnTPP, without the formation of aggregates, was efficiently incorporated into stable liposomes. Based on this, they claimed that hydrophobic porphyrin-derived structures with less tendency to form aggregation than ZnTPP can be efficiently incorporated into liposomes, and be useful for clinical applications [62].

ALA is the prodrug for the production of the photosensitiser PPIX in tumour cells. ALA-PPIX has been used for the treatment of a wide range of tumours. However, because ALA is highly hydrophilic, tissue penetration is limited. A liposomal carrier has been proposed to improve ALA tissue penetration. Results from the literature are still controversial. Early studies by Fukuda *et al.* compared PPIX synthesis in tumour and normal tissues using free and liposome-encapsulated ALA by subcutaneous, intraperitoneal and intratumoural administration, and found that PPIX formation was greater when liposome-encapsulated ALA was used [63,64]. Employing murine tumour culture technique, Casas *et al.* found that encapsulation of ALA in liposomes made of neutral phospholipid phosphatidylcholine, the same system used by Fukuda, did not increase tumour PPIX synthesis and PDT-induced tissue damage as compared with free ALA, although electronic microscopic study did indicate the endocytosis of liposomal ALA into the cells [65]. In a murine subcutaneous adenocarcinoma model, the same group studied the effects of various delivery systems (saline lotion with and without dimethylsulfoxide [DMSO], cream, liposomes and petroleum jelly) on ALA penetration and PPIX synthesis after topical application. Again, liposomal ALA showed less PPIX production than ALA in saline lotion, alone or with 10% DMSO [66]. However, Pierre *et al.* developed a liposomal delivery system for ALA with a lipid composition similar to the mammalian stratum corneum (stratum corneum lipid liposomes) in order to optimise skin delivery. This liposomal formulation included ceramide (50%), cholesterol (28%), palmitic acid (17%) and colesteryl sulfate (5%). Their results indicated that the delivery of ALA via stratum corneum lipid liposomes was deep enough for skin cancer treatment [67]. The discrepancy of these results may be due to the differing liposomal structures, sizes and routes of administration used in these studies.

5.2 Liposomal phthalocyanine photosensitisers

Liposomal phthalocyanine photosensitisers mainly include zinc, aluminium and silicon phthalocyanines with different increased degrees of sulfonation.

Liposomal Zn(II)-phthalocyanine (ZnPc) has been extensively studied for aspects of tumour cellular uptake [68], tissue distribution [69-74] and PDT efficacy [71-73]. Tumour cells *in vitro* take up DPPC liposomal ZnPc mainly through a passive diffusion-mediated pathway, which can be attenuated by the presence of serum and low incubation temperature [68]. The cellular uptake is fast and reaches a maximum within 8 h of incubation, presumably due to the hydrophobic nature of the compound. ZnPc is mainly located in the Golgi apparatus and the mitochondria of tumour cells [68]. In BALB/c mice

bearing transplanted MS-2 fibrosarcoma, unilamellar DPPC liposomal ZnPc is specifically transported by serum lipoproteins and cleared from the serum via the bile–gut pathway in a biphasic process [69]. Tumour tissues show a selective accumulation of liposomal ZnPC with maximum tumour/normal tissue ratio of 7.5 – 10 at ~ 24 h after administration [69,70,72]. It is believed that liposomal ZnPc is destabilised in the presence of lipoprotein and transfers ZnPc to lipoproteins, especially low-density lipoprotein (LDL), in circulation. The complex of LDL and ZnPc is selectively taken up by tumour cells through LDL receptor-mediated endocytosis as tumour cells generally have a high expression of LDL receptors [75]. The transfer of ZnPc from liposomes to serum lipoprotein in the circulation is further supported by the fact that the pharmacokinetic behaviour of liposomal ZnPc is very similar to that of parent molecule ZnPc [70]. As mentioned above, photosensitisers should be maintained in a monomeric state for maximum photosensitising efficiency. Isele *et al.* reported that ZnPc liposomes prepared with different ratios of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1,2-dioleoylphosphatidylserine significantly influenced ZnPc aggregation states [76]. Aggregation of ZnPc in liposomes was found to increase drug plasma clearance due to enhanced opsonisation effect, and, therefore, lowered the maximal drug concentration in tumour tissue.

Similar to liposomal ZnPc, some other phthalocyanine photosensitisers, including Ge(IV) phthalocyanine (GePc) [77], Zn(II) 2,3-naphthalocyanines [78] and Si(IV)-naphthalocyanine (SiNc) [24,26,79,80], are also formulated in DPPC liposomes to maintain a monomeric state for drug delivery. Most of these liposomal photosensitisers are found to be transported by serum lipoproteins, in particular LDL, and believed to be selectively internalised into tumour cells via receptor-mediated endocytosis. This pathway is the leading mechanism encouraging the formulation of photosensitisers in lipid carriers such as liposomes. However, the generalisation of this pathway is controversial [81]. For example, > 95% of SiNc incorporated into DPPC liposomes was associated with serum lipoproteins, 57.8% of which was bound to LDL. A ratio of tumour versus peritumoural normal tissue > 10 was obtained at 12 – 48 h after administration in Balb/c mice with MS-2 fibrosarcoma. [80]. However, the same liposomal SiNi did not show selective tumour distribution in B16 pigmented melanoma subcutaneously transplanted in C57BL mice [79], and formulation of SiNi in the Cremophor® (BASF) emulsion delivery system still caused no significant increase in tumour selectivity [24].

To improve the selectivity of PDT, targeted liposomes encapsulating the water-soluble photosensitive dye sulphonated aluminium phthalocyanine (AlSPc) were prepared by linking liposomes to a targeting monoclonal antibody [82]. Antibody-dependent photocytotoxicity was only observed in antigen-expressing cells and was proportional to the number of antigens on the cells, the AlSPc concentration and the light dose. Targeted liposomes were compared with aluminium tetra-3 sulfonyl chloride phthalocyanine directly conjugated with the same antibody for PDT efficacy. At equal molar AlSPc doses, phototoxicity was similar for the two conjugate types, but at equal molar antibody doses, the liposomal conjugate was ≤ 13-fold more effective. This study suggests that liposomal formulation has a much higher drug payload than the direct conjugate [83]. The folate receptor-mediated pathway has also been explored for selective photosensitiser delivery and tumour targeting. A water-soluble photosensitiser chloroaluminium phthalocyanine tetrasulfonate (AlPcS4) was formulated in folate-diplasmenylcholine (1,2-di-O-(Z-1′-hexadecenyl)-sn-glycero-3-phosphocholine; DPPlsC) liposomes and tested for phototoxicity. It was found that AlPcS4/DPPlsC:folate liposomes were substantially more phototoxic to folate-deficient KB cells than free AlPcS4 [46]. As many tumour cells overexpress the transferrin receptor, a targeted liposomal formulation (Tf-Lip-AlPcS4) was prepared by conjugating transferrin to PEG–liposomes, entrapping a water-soluble sensitiser AlPcS4. A phototoxicity study in HeLa cells indicated that Tf-Lip-AlPcS4 was 10-times more photocytotoxic than free AlPcS4, and Lip-AlPcS4 displayed no photocytotoxicity. The high photocytotoxicity of Tf-Lip-AlPcS4 was shown to be the result of a high intracellular drug concentration, which could be inhibited dramatically by incubating the conjugate with a competing concentration of transferrin [43]. The same liposomal delivery system was recently used to target AlPcS4 to bladder tumour cells, and encouraging results were also confirmed in animal experiments [44].

5.3 Liposomal chlorin photosensitisers

This category of liposomal photosensitisers mainly includes benzoporphyrin derivative, chlorin e6 trimethyl ester (Ce6) and pheophorbide photosensitisers.

Benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin) was formulated in the unilamellar liposomes composed of DMPC and egg phosphatidylglycerol [84]. Use of these fluid lipids enables fast drug release in the circulation, and the hydrophobic nature of BPD-MA prompts redistribution to lipoproteins. Liposomal BPD-MA was shown to have comparable tumour and normal tissue distribution with the free drug, although the liposomal drug appeared to enter tissues more rapidly and to be cleared more rapidly [84]. However, liposomal BPD-MA was shown to be more effective in tumour damage than free BPD-MA when PDT was performed at 3 h following intravenous drug administration. The difference in PDT efficacy probably results from liposomal formulation allowing more BPD-MA to be associated with lipoproteins so that tumour cells can effectively take up the drug [84]. Liposomal BPD-MA is also able to deliver BPD-MA to proliferating endothelial cells overexpressing LDL receptors [85]. This BPD-MA liposomal formulation (Visudyne) has received regulatory approval for the treatment of AMD and is the only liposomal photosensitiser approved for clinical application [86]. To improve tumour accumulation of BPD-MA, Oku *et al.* prepared long-circulating liposomes composed of DPPC, POPC, Chol, BPD-MA and palmityl glucuronide

(10:10:10:0.6:5 as a molar ratio) [87]. Differing from previous conventional BPD-MA liposomes, this liposomal system is coated with a hydrophilic moiety, palmityl glucuronide, to extend plasma circulation time in order to increase tumour accumulation. As expected, long circulating BPD-MA liposomes cause an 80% tumour cure, whereas conventional liposomes and free drugs only obtain a 20% tumour cure following the same treatment in the Meth A tumour model. The same group recently prepared another long circulating liposome [88]. Liposomes were composed of DPPC, POPC, Chol, dipalmitoylphosphatidylglycerol (DPPG) and BPD-MA (10:10:10:2.5:0.3 as a molar ratio) and coated with PEG. They found that long-circulating PEG–liposomes led to several times more tumour uptake of BPD-MA than the conventional liposomes without PEG modification at 3 h after administration. Surprisingly, PEG–liposomes had no significant tumour inhibition effect, whereas conventional liposomes with much less drug were able to inhibit tumour growth. This result indicates that a high drug level in the tumour tissue does not simply mean high antitumour efficacy. The ultimate goal of a tumour drug delivery system is to enhance intracellular drug uptake. As the plasma membrane is generally negatively charged, positively charged particles/molecules can adhere to membrane plasma by electrostatic attraction. Based on this principle, Oku and colleagues developed BPD-MA polycation liposomes (PCLs) to target endothelial cells [89-91]. Cetylated polyethylenimine (cetyl-PEI) was used as a cationic polymer in the preparation of PCLs, which includes DPPC, Chol, DPPG, cetyl-PEI and BPD-MA (20:10:5:1.75:0.3 in molar ratio). BPD-MA PCLs were stable in serum and showed reduced redistribution from liposomes to serum lipoproteins as compared with unmodified control liposomes. Nevertheless, BPD-MA PCLs showed enhanced cellular uptake and phototoxicity in human vascular endothelial cell lines ECV304 and human umbilical vein endothelial cells in comparison with a unmodified control liposome [89,91]. It is not yet clear whether this new BPD-MA liposomal preparation will improve clinical AMD treatment using Visudyne.

Liposomal formulation was also used to deliver pheophorbide photosensitisers. Mayhew *et al.* also prepared both conventional and long-circulating liposomes (modified with monosialoganglioside) to encapsulate the photosensitising agent methylpheophorbide-a-(hexyl-ether) (MPH) [92]. Although not stable in circulation, water-insoluble MPH could be incorporated in multilamellar liposomes for delivery. Tumour response to PDT was significantly greater for the liposomal MPH than for MPH solubilised in Tween 80. Surprisingly, there was no difference in tumour uptake between liposomal and Tween 80 formulations of MPH. Bergstrom *et al.* developed pheophorbide: a liposome coated with a monoclonal antibody directed against a bladder tumour cell line [93]. Targeted liposomes were localised in the lysosomes and incubation with a monoclonal antibody inhibited cellular uptake in MGH-U1 human bladder cells, thus indicating specific cellular uptake. Pheophorbide-a loaded, targeted liposomes were shown to be photocytotoxic towards MGH-U1 cells at a concentration as low as 500 ng/ml.

A potent, long-circulating liposomal formulation was recently developed to encapsulate the lipophilic sensitiser Ce6 [94]. Ce6 long circulating liposomes, composed of Ce6, dilauroylphosphatidylcholine, dioleoylphosphatidylethanolamine, and DSPE-*N*-PEG-2000 (23:46:46:10 in molar ratio), showed 80% lethal dose (LC_{80}) values of ≤ 53-times lower than that of Ce6 sodium salt in several human gastric cancer cell lines. PDT with Ce6 liposomes led to ~ 98% tumour cure in HSC-45 human gastric tumours in nude mice. To effectively deliver the photosensitiser 2,3-dihydro-5,15-di(3,5-dihydroxyphenyl) porphyrin (SIM01), Bourre designed DMPC liposomes to incorporate this new diphenylchlorin photosensitiser, and studied the pharmacokinetic behaviour and photodynamic effectiveness in human HT29 adenocarcinoma xenografted in nude mice [95]. Pharmacokinetic study indicated that maximum tumour concentration occurred at 12 h after injection for both liposomal SIM01 and free SIM01, with a higher tumour uptake for the liposomal formulation. In agreement, PDT with liposomal SIM01 was more effective than the free drug.

5.4 Liposomal bacteriochlorin photosensitisers

The importance of maintaining the photosensitiser in a monomeric state in the delivery system was studied by Damoiseau *et al.* using bacteriochlorin a (BCA). As the efficiency of the photosensitising effect is dependent on the quantum yield of triplet state and oxygen consumption to generate singlet oxygen, the authors measured the triplet state quantum yield [96] and oxygen consumption [97] of BCA in different delivery systems. In PBS, BCA was in a state of monomer–dimer equilibrium. The triplet-state yield was only 0.095 and the oxygen consumption was related to the BCA monomer concentration in solution. Incorporation of BCA in DMPC liposomes, by promoting the monomerisation of BCA, increased the oxygen consumption ninefold in comparison with the value in PBS, and the yield of triplet state was increased to 0.4. Irrespective of the dye concentration, cell survival of WiDr tumour cells after PDT was always lower when the liposomal formulation was used.

5.5 Other liposomal photosensitisers

The liposomal delivery system has also been used to formulate synthetic porphycene photosensitisers. For instance, palladium(II)-tetraphenylporphycene was formulated in DPPC liposomes [98,99]. Toledano *et al.* studied the PDT efficacy of four tetrakis (methoxyethyl) porphycene derivatives encapsulated in DPPC liposomes [100]. Fluorescence-quenching experiments demonstrated that porphycenes are located in the liposome bilayer at various depths, depending on the hydrophilicity of the porphycene side chains. *In vitro* PDT damage depended on the location of porphycenes. Porphycenes located deep inside the bilayer did not cause damage *in vitro*, but still caused *in vivo* damage to blood vessels

of the chorioallantoic membrane of the fertilised chick embryo. Based on this observation, the authors concluded that liposomes were fused with the cell membrane to release the photosensitiser *in vivo*, whereas *in vitro* the sensitiser was transferred to cells from liposomes only through contact. An interesting pH-sensitive liposomal formulation was prepared by Aicher *et al.* to deliver hydrophobic photosensitiser 9-acetoxy-tetra-n-propylporphycene [101]. As tumour tissues generally have lower pH values than normal tissues, pH-sensitive liposomes may achieve targeted drug release in tumour tissues. The authors compared the photocytotoxicity between pH-sensitive liposomes composed of phosphatidylethanolamine and cholesteryl hemisuccinate, and pH-insensitive liposomes consisting of phosphatidylcholine and cholesteryl hemisuccinate in human bladder carcinoma cells, and found that pH-sensitive liposomes were significantly more phototoxic than pH-insensitive liposomes.

To specifically deliver photosensitiser to tumour cells overexpressing transferrin, Derycke *et al.* encapsulated the photosensitiser hypericin into transferrin-conjugated PEG–liposomes [42]. The same delivery system has been successfully used to selectively deliver water-soluble photosensitiser AlPcS4 to transferrin-overexpressing HeLa and AY-27 cells [43,44]. However, hypericin encapsulated in transferrin-conjugated PEG–liposomes did not show targeted delivery and photocytotoxicity as compared with nontargeted PEG–liposomes or free hypericin. This is a good example illustrating that the selection of the correct drug for liposomal delivery is essential to the success of this delivery system. As hypericin is hydrophobic, it will mainly associate with the lipid layer of liposomes and rapidly redistribute to the plasma proteins. A substantial leak of hypericin out of the liposomes due to the limited embedding stability makes targeted liposomes behave like a free drug [42]. In contrast, the hydrophilic property of AlPcS4 enables the stable entrapment in the aqueous compartment of targeted liposomes, and consequent cellular binding and internalisation into tumour cells.

Hypocrellin B was incorporated into egg la-phosphatidylcholine liposomes for drug delivery [102]. The quantum yield of singlet oxygen generated by hypocrellin B in egg la-phosphatidylcholine liposomes was the same as in chloroform (~ 0.7), indicating a monomeric state in liposomes. The analogue hypocrellin A (HA) was also formulated in liposomes [103]. Wang *et al.* compared tissue distribution and PDT effect between liposomal HA and HA dissolved in DMSO saline solution in S-180 tumour-bearing mice. Liposomal delivery improved the selective accumulation of the sensitiser in tumours with higher ratios of tumour-to-muscle and tumour-to-skin than the DMSO saline system, although liposomal HA showed a slower pharmacokinetic behaviour. Increased tumour uptake of liposomal HA translated to a superior PDT efficacy as compared with HA in DMSO saline solution.

An interesting light-sensitive liposomal delivery system was developed by Bisby *et al.* to load the photosensitiser acridine orange [104]. This liposomal formulation was composed of DPPC and a photochromic lipid, 1,2-(4′-n-butylphenyl)azo-4′-(γ-phenylbutyroyl)-glycero-3-phosphocholine, which isomerises on exposure to near-ultraviolet light with resulting changes in membrane permeability to solutes. Once exposed to a single ultraviolet laser pulse, acridine orange was rapidly released from liposomes containing 1,2-(4′-n-butylphenyl)azo-4′-(γ-phenylbutyroyl)-glycero-3-phosphocholine.

6. Expert opinion and conclusions

Liposomal delivery has become a common practice in PDT and has been used to formulate many types of photosensitisers with different chemical structures and physicochemical properties. As an example, liposomal verteporfin has been successfully translated for clinical application and is approved as a first-line treatment of AMD. The delivery of photosensitising agents in liposomes may serve many functions, such as to:

- solubilise hydrophobic photosensitisers
- maintain drugs in a monomeric state for systemic (intravenous) administration and improved photochemical properties
- enhance delivery of the photosensitising agents in absolute terms by increased circulation times and, in some cases, better uptake
- increase tumour selectivity

However, most current liposomal photosensitisers are contained in conventional liposomes that have short circulation times due to significant reticuloendothelial system uptake. The hydrophobic nature of most photosensitisers results in liposomal formulations with low entrapment stability because of rapid redistribution from liposomes to plasma proteins. Thus, current applications focus primarily on using liposomes as a means to formulate photosensitising agents so that they can be administrated intravenously, rather than employing them as delivery systems to increase tumour selectivity. In this context, the difference between liposomal formulation and simple preparations using emulsifying agents is not significant. However, in the future, as liposomes with different structures and sizes are adopted in PDT, they may be used as targeting agents as well as a solubilising strategy. It is important to note that not all photosensitising agents are suitable for liposomal delivery. The choice of photosensitiser for liposomal delivery should be made with a careful balance between plasma stability and drug availability, taking into consideration the potential application and the limitations of existing liposomal technology. In recent years, more and more advanced liposomal formulations for encapsulating drugs have been developed. These liposomes typically have a long circulating time and are designed to target specific biological targets. Some of them are even equipped with triggered release mechanisms to ensure enhanced selective drug release. This is certainly the future direction of liposomal photosensitising agents; in initial studies the effectiveness of such liposomal photosensitisers has been evaluated mainly in

tumour cells *in vitro*. *In vivo* animal testing has been limited; more *in vivo* studies are clearly needed in order to obtain enough evidence and enthusiasm for potential clinical trials of these agents. Detailed pharmacokinetic studies of liposomes with different structures and sizes may help point to specific applications. For example, it may turn out that a set of diseases are best treated with cationic, large liposomal preparations whereas a different disease type may be more appropriately targeted with small, neutral formulations of the same photosensitiser. Also useful would be a study of liposomal photosensitisers in appropriate animal models, as the distribution, pharmacokinetics and toxicities may be impacted significantly by the models used. Finally, a detailed picture about the trafficking of liposomes, of the interactions with plasma and tissue components, and the final breakdown and drug release, is still not clear. Understanding the mechanisms behind these important processes will be crucial for the safe, rational and effective use of this delivery system.

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