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Invited Review

State of the art in the delivery of photosensitizers for photodynamic therapy

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Abstract

In photodynamic therapy, one of the problems limiting the use of many photosensitizers (PS) is the difficulty in preparing pharmaceutical formulations that enable their parenteral administration. Due to their low water solubility, the hydrophobic PS cannot be simply injected intravenously. Different strategies, including polymer–PS conjugation or encapsulation of the drug in colloidal carriers such as oil-dispersions, liposomes and polymeric particles, have been investigated. Although these colloidal carriers tend to accumulate selectively in tumour tissues, they are rapidly taken up by the mononuclear phagocytic system. In order to reduce this undesirable uptake by phagocytic cells, long-circulating carriers that consist of surface modified carriers have been developed. Moreover, considerable effort has been directed towards using other types of carriers to improve tumour targeting and to minimize the side effects. One of the approaches is to entrap PS into the lipophilic core of low-density lipoproteins (LDL) without altering their biological properties. The LDL receptor pathway is an important factor in the selective accumulation of PS in tumour tissue owing to the increased number of LDL receptors on the proliferating cell surface. Specific targeting can also be achieved by binding of monoclonal antibodies or specific tumour-seeking molecules to PS or by the coating of PS loaded carriers.

Keywords: Photodynamic therapy; Photosensitizers; Drug delivery systems; Passive targeting vehicles; Active targeting systems; Review

1. Introduction

Photodynamic therapy or PDT is a promising treatment for a variety of oncological, cardiovascular, dermatological, and ophthalmic diseases [1]. PDT is based on the use of photosensitizers (PS), which are preferentially taken up and/or retained by diseased tissue. Upon photoactivation with visible light at the appropriate wavelength, the generation of cytotoxic species, such as reactive singlet oxygen leads to irreversible destruction of the treated tissues [2]. Compared to current treatments including surgery, radiation therapy and chemotherapy, PDT offers the advantage of an effective and selective method of destroying diseased tissues without damaging surrounding healthy tissues.

The observation of tissue photochemical sensitization was first described by Raab in 1900 [3]. The PDT was subsequently investigated by von Tappeiner et al. [4]; they used topical administration of eosin combined with sunlight to treat skin tumour patients. Based on earlier work in the mid-1950s, Lipson et al. [5] synthesized a hematoporphyrin derivative (Photofrin® I, HpD) in order to enhance the tumour accumulation of the dye and subsequently, this PS was used as a fluorescent marker for cancer diagnosis. During the 1970s, research [6,7] led to an increased understanding of the cytotoxic mechanism of PDT [8]. Preclinical studies using HpD performed by Dougherty et al. [6,7] on 25 patients showed that a partial or complete tumour necrosis was obtained in 111 of 113 treated tumours. Today, Photofrin® II, a purified HpD derivative, is the most commonly used PS and it is the only drug approved by the Food and Drug Administration for the treatment of superficial bladder in Canada and early lung and advanced oesophageal cancers in the Netherlands and Japan. However, these active substances named first-generation PS suffer from several drawbacks [2]. Firstly, they are a complex mixture of several partially unidentified porphyrins and they show a poor selectivity in terms of target tissue/healthy tissue ratios (PDT selectivity index). Secondly, their low extinction coefficients require the administration of relatively large amounts of drug to obtain a satisfactory phototherapeutic response. Furthermore, the

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absorption maximum is at a relatively short wavelength (630 nm) leading to a poor tissue penetration of light. Finally, they present a high accumulation rate in skin, which induces a prolonged cutaneous light ultrasensibility lasting for up to 6–8 weeks after PDT treatment. During this post-treatment period, patients have to stay out of sunlight to avoid a severe sunburn reaction [9].

The problems encountered with Photofrin® II, have led to the development of new molecules called second generation PS such as porphyrin derivatives [10], phthalocyanines [11,12], naphthalocyanines and chlorins [13–15]. These new compounds have the advantage of being pure and well characterized. They are effective generators of singlet oxygen and have a strong absorption peak in the range of 650–800 nm, wavelength at which light penetration in tissue is enhanced. In addition, their high selectivity for diseased tissues leads to a better target to healthy tissue ratio and their relatively fast elimination from the body shortens side effects. However, most of these PS are hydrophobic. This lipophilic nature may be an important factor affecting the preferential accumulation in cellular hydrophobic loci since these molecules must be able to get into cells by crossing lipid membranes [16]. However, due to their minute solubility in water, intravenous treatment is greatly hampered. Thus, it is necessary to develop suitable delivery systems such as oil-dispersions, liposomes, polymeric particles (nanoparticles and microparticles) or hydrophilic polymer–PS conjugates. Moreover, to enhance the specific uptake by targeted tissue and improve PDT efficiency, other concepts using PS complexed with serum lipoproteins or conjugated with specific monoclonal antibodies or other specific tumour-seeking molecules have been developed in recent years.

The ideal drug delivery system should enable the selective accumulation of the PS within the diseased tissue and the delivery of therapeutic concentrations of PS to the target site with little or no uptake by non-target cells. The carrier must also be able to incorporate the PS without loss or alteration of its activity. In view of the high probability of repetitive dosing schedules, the system must also be biodegradable and have little or no immunogenicity [17]. Another reason for using vehicles is to provide an environment where the photosensitizer can be administered in a monomeric form. In fact, due to their chemical structure, most photosensitizers tend to aggregate in aqueous media as a result of the propensity of the hydrophobic skeleton to avoid contact with water molecules [12]. This state is one of the determining factors, which can hinder the efficacy of the drug in vivo by decreasing its bioavailability and limiting its capacity to absorb light [11,18,19].

This paper covers the different approaches currently used in the formulation of PS that enable the parenteral administration and passive targeting namely, liposomes, oil-dispersions, polymeric particles and hydrophilic polymer–PS conjugates. The strategies to deliver specifically the PS to diseased tissues using the target tissue receptors or antigens are also discussed. Although PDT has many therapeutical applications (for other review articles see Refs. [20–22]), this article is essentially focused on the tumour treatment.

2. Mechanisms of PDT cytotoxicity

The photodynamic activity of PS is based on photooxidative reactions, which induce multiple consecutive biochemical and morphological reactions [23,24]. Generally, the PDT procedure consists in administering the photosensitizer formulation usually systemically by means of an intravenous injection. Then, the diseased tissue is illuminated with light at an appropriate wavelength and energy dose at an appropriate time after the injection corresponding to a maximum photosensitizer accumulation in target tissue. Following the absorption of light, the photosensitizer, initially at a ground state (PS^0), is activated to a short-lived excited state ($^1PS^*$) that may convert to a long-lived triplet state ($^3PS^*$) (Fig. 1). The main role of the singlet state in the photosensitization process is to act as a precursor of the triplet state. This triplet state is the photoactive state, which may generate cytotoxic species by undergoing two main reactions. Type I reactions can produce free radicals or superoxide ions resulting from hydrogen or electron transfer. Type II reactions involve the interaction between oxygen and the triplet state of the sensitizer to mediate formation of singlet oxygen, which is generally believed to be the main cytotoxic species in PDT. These reactive oxygen species are responsible for irreversible damage to various cell membranes including plasma, mitochondria, lysosomal and nuclear membranes and of protein modifications. The complete eradication of diseased tissue is believed to be largely due to the shutdown of tissue vasculature that provokes blood stasis, thus starving the diseased tissue of oxygen and nutrients. Ultimately, the target tissue is eradicated with either little or no damage to surrounding normal tissues.

The PDT efficiency depends on the chemistry of the PS, the pharmaceutical formulations, the physical localization and the amount of PS in treated tissue, time of activation with light, the light doses and the amount of oxygen. A schematic presentation of the photophysical reactions is shown in Fig. 1.

3. Passive targeting vehicles

Due to the fact that they exploit the natural distribution pattern (passive diffusion and phagocytosis processes), liposomes, oil dispersions, biodegradable polymeric particles and hydrophilic polymer–PS conjugates are considered as passive targeting systems [17]. Several studies [25–27] have shown that the selective accumulation in target tissues such as tumours or neovasculature of these

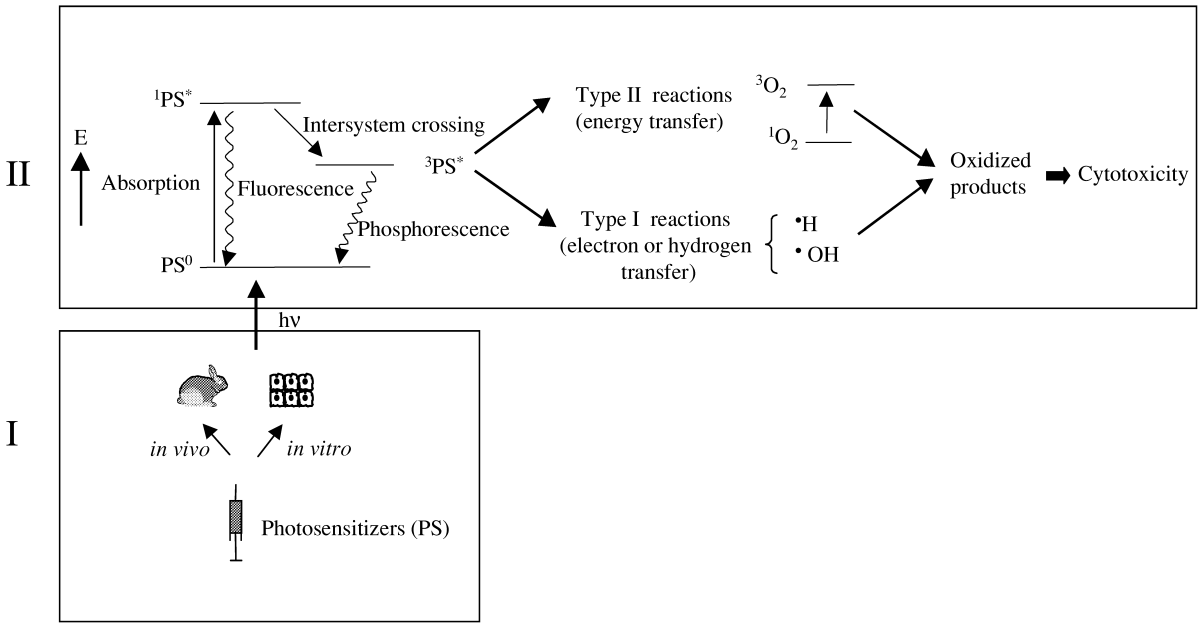

Fig. 1. Mechanism of PDT cytototoxicity: PS administration (step I) following to photophysic reactions represented by modified Jablonski diagram (step II) (vibrational levels omitted).

targeting carriers is due to the phenomenon known as ''enhanced permeability and retention effect''. In fact, the leaky vasculature leading to incomplete endothelial barrier, the poor lymphatic drainage resulting from underdevelopment of the lymphatic system or lymphatic obstruction and an increased vessel permeability are some mechanisms which provide an opportunity for these particles to reach their target site by simple diffusion. Indeed, the enhanced capillary permeability of many tumours seems due to a perturbation of the endothelial cell integrity resulting from the activity of a tumour-secreted factor or inflammatory process. These phenomena may enhance the extravascular transport of large particles by a physical separation of endothelial cells. In addition, lymph channels, which recover extravasated molecules, are poorly developed in tumour tissue. The passive targeting is illustrated by the data from biodistribution, pharmacokinetics, and phototoxicity studies discussed in the following sections including liposomes, oil-dispersions (Table 1), polymer particles and hydrophilic polymer–PS conjugates (Table 2).

3.1. Liposomes

Liposomes are multilamellar or unilamellar phospholipidic submicroscopic vesicles which allow the incorporation of lipophilic and hydrophilic drugs in their matrix because of their particular nature (i.e. concentric lipid bilayers encapsulating aqueous compartments). Their

Table 1
Biodistribution of PS delivered by liposomal formulations or oil-dispersion vehicles

PS	Vehicle	Dose (mg/kg)	Targeted tumour (Host)	Host	Maximal tumour levels (mg/kg tissue)	Tumour/healthy tissue ratio	Ref.
BPD-MA	DPPC liposomes	4	M1 rhabdomyosarcoma	DAB/2 mice	1.88	19.2	[33]
ZnPc	POPC/OOP liposomes		Meth-A sarcoma	Balb/c mice			[35]
100% MF[a]		0.25			0.67	NA	
80% MF		0.25			0.2	NA	
Ge(VI)Pc	POPC/OOPS liposomes	0.76	MS-2 fibrosarcoma	Balb/c mice	0.74	4.35	[36]
		1.52			1.87	5.67	
Purpurin	CRM	0.0025	experimental urothelial	Rats	NA	NA	[41]
		0.005	tumour		NA	NA	
Ketochlorin	CRM	5	RIF tumour	C3H mice	3.5	NA	[45]
	Tween® 80	5			6.7	NA	
Meso Porphyrin	CRM	5	RIF tumour	C3H mice	2.35	NA	[44]

[a] % MF=percentage of monomeric form; NA=not assessed.

Table 2
Uptake and phototoxicity of PS delivered by polymer particles (NP, MS) or polymer conjugates (HPMA, PEG, PVAL)

PS	Carrier	Dose	Target	Comments	Ref.
$ZnPcF_{16}$	PEG-coated NP	1 μM kg^{-1}	EMT-6 mammary tumours	Maximum tumour concentration was 4.01 μg g^{-1} tissue at 48 h p.i. and tumour/skin and tumour/muscle ratios were 3.5 and 10.8, respectively.	[67]
Ce_6	MS	0.2 μM	Trabecular meshwork cells	Optimal rate of phagocytosis was 50 MS per cell. At 20 J/cm^2, 95% cell death against 30% with free dye.	[68]
Ce_6	MS	0.43 μM	MGH-U1 bladder carcinoma cells	Number of MS per cell was 20 and evident morphological damage after 5 J/cm^2. 95% cell death 24 h after irradiation at a radiant exposure of 20 J/cm^2.	[68,146]
MCe_6	HPMA	1–10 μM	Human ovarian carcinoma cells	IC_{50} for HPMA-MCe_6 was about 100 higher compared to free MCe_6.	[71]
AlCPc	PEG PVAL	0.25–10 μM kg^{-1}	EMT-6 tumour transplanted on Balb/c mice	AlCPc-PVA showed an extented plasma half-life (6.8 h) as compared to CRM formulation (2.6 h) and AlCPc-PEG, lower retention by liver and spleen and higher tumour/normal tissues ratios. All the three formulation were highly phototoxicity.	[81]
m-THPC	PEG	30 mg/kg	Ovarian cancer transplanted on rat	At 8 days after dye injection, tumour/normal tissues ratio was 40:1.	[80]

main components (phospholipids and cholesterol) are materials also existing in the body in high amounts and may provide a good biocompatibility [28].

In PDT studies, liposomal preparations are currently used as effective delivery systems in experimental studies and in clinical trials. The beneficial effect of these colloidal carriers in PDT has been described by a number of investigations, which have shown their advantages over other formulations such as simple aqueous dispersions of the drugs [29–31]. For instance, Jori et al. [32] have studied the pharmacokinetic profile of hematoporphyrin encapsulated into small unilamellar dipalmitoylphosphatidyl-choline (DPPC) in comparison to dye dissolved into phosphate buffer saline (PBS). The in vivo study in MS-2 fibrosarcoma-bearing mice has shown that despite a slow rate of dye accumulation in the tumour, the maximum concentration was higher than with the PBS formulation. Using the benzoporphyrin derivative monoacid ring A (BPD-MA) as photosensitizer, Richter et al. [33] also compared the biodistribution and clearance of an unilamellar liposomal formulation (L-BPD-MA) versus a dimethyl sulfoxide solution (DMSO-BPD-MA) and a PBS solution (PBS-BPD-MA). After intravenous injection of 4 mg kg^{-1} body weight of the dye into M1 rhabdomyosarcoma-bearing DAB/2 male mice, L-BPD-MA was rapidly and significantly accumulated into the tumour tissue. The maximum concentration of L-BPD-MA in tumour tissue was obtained 15 min post-injection (p.i.) against 3 h p.i. for both aqueous formulations. The highest tumour/tissue ratios were obtained at 24 h p.i. (19.2 and 12.6 for L-BPD-MA and DMSO/PBS-BPD-MA, respectively). The results from PDT treatment at 150 and 210 J/cm^2 showed that the liposomal formulation was significantly more efficient. The doses inducing 50% of tumour free animals were 1.9 mg/kg at 150 J/cm^2 for the liposomal formulation and 3.1 mg/kg at 210 J/cm^2 for both aqueous formulations.

One of the expectations when incorporating PS into liposomes is to maintain the PS monomeric state since monomers are known to increase the oxygen consumption and to be more efficiently photosensitizing than aggregate species [31,34]. Isele et al. [35] have developed an organic solvent dilution procedure to produce monomeric zinc phthalocyanine (ZnPc) entrapped in stable lyophilized liposomes, consisting of a mixture of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1,2-dioleoylphosphatidylserine (OOPS) (9:1). The influence of this factor on in vivo dye behaviour was discussed in a subsequent study [19] where a correlation of different degrees of ZnPc aggregation within liposomes (expressed as percentage monomeric fraction (% MF)) with pharmacokinetic and biodistribution parameters was established. Meth-A sarcoma-bearing female Balb/c mice were used as animal model. In fact, after injection of 0.25 mg/kg body weight of each aggregation state, the clearance rate of the dye from the plasma as well as the uptake by the liver increased with decreasing % MF of ZnPc. The tumour uptake proportionally increased with increasing % MF of ZnPc (e.g., 48 h p.i., 670 ng/g tissue for 100% MF as opposed to 200 ng/g for 28% MF). The large fraction of ZnPc that aggregated in the liposome bilayers appeared to enhance liposome opsonization and their uptake by the macrophages leading to lower concentration of the dye in tumours. This aggregation state may probably affect the transfer ability of ZnPc liposomes to lipoproteins, which are important endogenous carriers of the PS to the tumour [36]. It has been reported that in the bloodstream, liposomes may serve as donors for the direct transfer of the drug to LDL [37,38]. According to Gregoriadis [39], the transfer phenomenon may be due to a destabilization of liposomal membrane by the high-density lipoproteins (HDL) following an increase in liposomal permeability. The lack of cholesterol in the liposome bilayer seems to be a major factor, which intensifies the membrane destabilization phenomenon [40].

In order to improve the transport of liposomes by the LDL in the bloodstream and their accumulation in tumours, Segalla et al. [36] made a complex associating germanium phthalocyanine (Ge(IV)Pc) and two axially ligated cholesterol moieties. At 24 h p.i. in MS-2 fibrosarcoma-bearing Balb/c mice, the Ge(IV)Pc liposomal formulations were effectively accumulated in tumour tissue via lipoprotein pathways (i.e. 0.74 and 1.87 μg/g tumour tissue, with tumour/peritumoral tissue ratio of 4.35 and 5.67 for Ge(IV)Pc injected doses of 0.74 and 1.52 mg/kg, respectively). Thus, doubling the Ge(VI)Pc dose increased tumour accumulation at 24 h p.i. by a factor of 2.5 with a low dye content in muscle and skin, associated with a massive photodynamic destruction of the tumour.

Liposomes are currently used as PS vehicle in clinical trials to successfully treat other diseases such as choroidal neovascularization [41–43]. Recently, the liposomal BPD-MA (Visudyne®, QLT, Canada) was approved in Switzerland and USA for the treatment of the wet form of age-related macular degenerescence, which is characterized by the formation of choroidal neovasculature.

Other colloidal systems thoroughly tested in PDT, are dispersion systems containing an emulsifying agent or micelle systems. In such systems, PS solubility is considerably increased.

3.2. Oil-based dispersions or micelle systems

In fact, the dispersions containing Cremophor-EL (CRM), a non-ionic poloxyethylated castor oil as emulsifying agent is an other alternative to resolve the solubility problems of hydrophobic PS. They are used in the preparation of parenteral formulations. PDT studies using this vehicle were carried out by Selman et al. [44] for the treatment of an experimental transplantable bladder tumour grafted in rats. Using purpurin derivatives as photosensitizers, the cytotoxic assays of the dye dispersed into CRM

have shown a tumour regression at significantly lower doses than HpD. Unfortunately, no assessment of tumour/normal tissues ratios was carried out in order to predict skin photosensibility. In a subsequent PDT study, using the same tumour model, the same research group [45] has shown that when using tin etiopurpurin ($SnET_2$) at the dose of 1 mg/kg, both the CRM dispersion and dye loaded DPPC liposomes presented the same tumour response, i.e. 100% tumour cure, whereas at lower doses, the higher phototoxic activity was only obtained with the CRM formulation. These two vehicles loaded with bis(methyl-oxyethyleneoxy)silicon-phthalocyanine (SiPc) were also tested on C57B1/6 mice bearing a transplanted Lewis lung carcinoma [46]. They exhibited a high efficacy and similar tumour response despite the difference in the SiPc accumulation in the tumour tissue (tumour concentration of SiPc embelled in CRM formulation is about two times higher than liposomes). Kessel et al. [47] have also performed a comparative study of the $SnET_2$ photobiological properties using ethanolic solution versus three types of vehicles, namely CRM, gamma-cyclodextrin (γ-CD) or molecusol (MOL) (a more water soluble cyclodextrin derivative). They found that the accumulation of the dye into L1210 cells was time and temperature dependent for CRM, γ-CD and MOL resulting in the localization of dye at cellular loci and highly effective photodamage. Among the three vehicles, CRM promoted a very efficient photodamage, which was correlated with intracellular concentrations of dye. On the other hand, $SnET_{2,}$ delivered by γ-CD, provided a greater tumoricidal response in a transplanted bladder tumour-bearing rat due to photodamage of tumour vasculature. Nevertheless, the lipoprotein binding studies [48] showed that after 24 h of in vitro incubation with dog serum, the level of $SnET_2$ bound to LDL depended on the nature of the lipid-based vehicle and followed the order CRM>γ-CD>DPPC liposomes. A preferential uptake of hydrophobic PS dispersed in CRM by tumour tissue was attributed to lipoprotein-mediated distributions [49,50]. In fact, the success of CRM was associated with the degradation of plasma lipoproteins, resulting in the appearance of a 'pseudo-LDL', a new species that has electrophoretic lipoprotein behaviour. This has been confirmed by Woodburn et al. [50] who compared the relative biodistribution of ketochlorin (C8KC) solubilized with CRM or with polyethylene sorbitan monoleate (Tween®80). As opposed to Tween®80, a high concentration of CRM (>0.03%) induced a change in electrophoretic behaviour of HDL which is converted into a product with the same specific gravity as LDL. Increasing the amount of CRM favoured the binding of the dye to LDL and reduced the binding to albumin. In vivo PDT studies carried out on RIF tumour-bearing mice treated with 5 mg/kg ketochlorin solubilized in the same vehicles showed that the higher PDT efficiency was attributed to CRM formulation. The maximum tumour concentrations were 6.7 μg/g tissue for CRM and 3.5 μg/g tissue for Tween®80. This treatment protocol induced a longer plasma persistence and significant accumulation of the dye in the RIF tumour cells. In subsequent studies [49], the same group has shown that CRM and non-ionic detergents like Tween®80 or Triton X100 have the same alteration effect on the electrophoretic behaviour of lipoproteins at minimal dose of 0.4 mg/ml. After administration of 5 mg/kg of mesoporphyrin solubilized in CRM or dissolved in 10 mM NaOH solution, the biodistribution of dye in RIF tumour-bearing mice showed that CRM improved the dye accumulation into the tumour associated with a clearance of dye from the circulation compared to NaOH solution. Maximal tumour uptakes of 2.77 μg/g and 2.35 μg/g were observed for CRM and NaOH, respectively. Kongshaug et al. [50], had similar results when studying the effect of two delivery systems, namely dimethyl sulfoxide (DMSO) and CRM on the interaction of $SnET_2$ with human plasma proteins.

In view of the experimental data, CRM dispersions can be regarded as a suitable vehicle for hydrophobic photosensitizers but acute anaphylactic reactions following parenteral administration of formulations containing CRM as emulsifying agent have been reported [51–54]. The presence of CRM is likely to be the cause of these reactions and the incidence is correlated with the dose and the infusion rate. The mechanism of this hypersensibility reaction was well described by Dorr et al. [46]. At this stage, it is still difficult to avoid completely these unacceptable side effects but they may be alleviated by slow rates of drug infusion and premedication with antihistamine and corticosteroids [52].

Recently, pH-sensitive polymeric micelles have been investigated as a promising alternative to CRM formulation for the vectorization of PS such as aluminium chloride phthalocyanine (AlClPc) [55–57]. The in vitro PDT of AlClPc incorporated into different types of polymeric micelles against EMT-6 mouse mammary tumour cells showed higher phototoxicity than AlClPc-loaded CRM formulation. However, in vivo, they showed similar efficacy than CRM formulation in terms of EMT-6 tumour regression and cure despite their fast clearance from the bloodstream and their accumulation in normal tissue with a lower tumour uptake [57].

Lipid emulsions containing phospholipds and soy-bean oil or Miglyol 812 as lipidic phase and 2.5% glycerol in distilled water as aqueous phase were also used as delivery system for benzochlorin derivatives [58].

3.3. Polymeric particles

Biodegradable nanoparticles, considered as an alternative to liposomes, have received tremendous attention as a possible means of delivering antineoplastic agents [59]. Their main advantages lie in a high drug loading and the possibility of controlling the drug release, a large variety of materials and manufacturing processes [60–62].

In the field of photodynamic therapy, few experimental

studies have been carried out with nanoparticles. Some investigations with hematoporphyrin [63], phthalocyanine [61] or meso-tetra(hydroxyphenylchlorine) (m-THPC) [64] loaded biodegradable nanoparticles have given encouraging results. Labib et al. [61] have encapsulated tetrasulfonated zinc phthalocyanine ($ZnPcS_4$) or aluminium naphthalocyanine into poly(isobutylcyanoacrylate) (PIBCA) or poly(ethylbutylcyanoacrylate) (PEBCA) nanocapsules or nanospheres. In a subsequent study [65] with cultured V-79 hamster cells as model, they found that the nanoencapsulation enhanced the cellular localization of the dyes providing a good in vitro phototoxic activity. Indeed, $ZnPcS_4$-loaded polyisohexylcyanoacrylate nanocapsules turned out to be more active than the free dye formulation. Likewise, other research groups [66,67] have also studied the potential incorporation of phthalocyanine derivatives into Food and Drug Administration approved polyesters such as poly(D,L-lactic acid) (PLA). A comparative photodynamic activity study using two different formulations of hexadecafluoro ZnPc ($ZnPcF_{16}$) (PLA nanoparticles and CRM dispersion) in EMT-6 tumour bearing mice was carried out by Allémann et al. [67]. At a dose of 5 μmol/kg, PDT provided tumour regression in 100% of mice treated with nanoparticle formulation against only 60% with CRM emulsion.

Other polymeric particles such as microspheres larger than 1000 nm were also investigated in PDT studies but the only reported studies concerned hydrophilic PS. Thus, using chlorin e_6, polystyrene microspheres conjugated (Ce_6-MS), Bachor et al. [66,68] found that Ce_6-MS led to a high phototoxic effect compared to the unconjugated dye at equivalent dose. After incubation on MGH-U1 human bladder carcinoma cells, the Ce_6-MS uptake by cells was 20 times higher than that of free dye. Ce_6-MS was 10 times more phototoxic leading to complete inhibition of the tumour cells. They also found a difference between intracellular localization of both formulations. Ce_6-MS were taken up by phagocytosis and localized within intracytoplasmic compartments while the unconjugated dye was in the cellular membranes. This is supported by a previous study carried out by Latina et al. [69] on phagocytic trabecular meshwork cells (TM cells) with the same dye. Despite these interesting results, in vivo applications of these microparticles are not appropriate since polystyrene particles are not biodegradable.

3.4. Polymer–PS conjugates

Interesting possibilities were opened by increasing the hydrophilicity of the PS through the coordination of the appropriate polymers including *N*-(2-hydroxypropyl)methacrylamide (HPMA) [70,71] to cationic, anionic or neutral poly-L-lysine [72,73], polyethylene glycol (PEG) (pegylation) [74–82] or poly(vinyl alcohol) (PVAL) [81]. Polymer-bound PS are under extensive investigations and have a promising PDT applications compared to free PS due to extended intravascular half-life, enhanced tumour selectivity and good PDT efficacy. HPMA copolymer-bound mesochlorin monoethylenediamine (CMA) exhibited a substantial increased tumour accumulation and drug efficacy. Indeed, it has been reported that the dose inducing 50% of inhibition of human ovarian carcinoma cell growth was 100 times higher for the conjugate compared to free drug [71].

The photodynamic potential of PEG and PVAL conjugated to AlClPc via an axial coordination bond was also evaluated in vitro and in vivo [81]. The biodistribution studies performed in EMT-6 tumour-bearing mice revealed that among AlClPc-loaded CRM dispersion and two polymeric conjugates, the most interesting pharmacokinetic profile was obtained with AlClPc–PVAL, which showed the longest plasma circulation time. AlClPc–PVAL also exhibited the lowest retention by the liver, spleen and lungs, the best tumour/skin and tumour/muscle ratios as well as the higest and most persistent tumour uptake. The addition of hydrophilic axial ligands to AlClPc did not reduce the PDT efficiency since the two water-soluble AlClPc–PVAL and AlClPc–PEG conjugates showed photodynamic activities similar or superior to that of the dye in CRM dispersion.

PEG was also bound to m-THPC (PEG–m-THPC). This conjugate has proven to be effective in inducing tumour growth delay at m-THPC equimolar doses [78] and even has shown to be more effective in inducing tumour necrosis in some tumour models with minimal normal tissue damage [83,84]. However, the benefit effect of PEG–mTHPC in treating rat liver tumour model seems to occur only at early times after drug administration [82]. In contrast, pegylated silicon naphthalocyanine showed poor tumour selectivity and no phototherapeutic activity in a MS-2 fibrosarcoma transplanted in Balb/c mice [85]. Indeed, it needs to be noted that in the litterature, PS are often conjugated with different PEG chain lengths, which makes it difficult to compare directly the results of these studies since the PEG chain length could influence the pharmacokinetics.

4. Long-circulating delivery systems

The therapeutic applications of intravenously administrated colloidal carriers have been limited due to the propensity of the mononuclunear phagocytic system (MPS) to rapidly remove these particles from the bloodstream. Consequently, the uptake of colloidal carriers or dye by target tissues turns out to be insufficient. The uptake by phagocytic cells is promoted by opsonization (i.e. the adsorption of plasma protein ligands susceptible of interacting with receptors situated on phagocytic cell surface) [25,86]. In vivo studies revealed that the opsonin-particle interaction depends on the physicochemical properties of the particles such as hydrophobicity, charge, size

and fluidity of the particle surface [87]. Several approaches for avoiding uptake of particulate carriers by MPS such as blockade of MPS by saturation [88] or surface modification (charge, hydrophilicity, steric stabilization) were considered. In cancer treatment, the blockade of MPS is not really desirable because peritoneal macrophages and natural killer cells are implicated in the host's antitumour immune activities, which control tumour development and spread. Thus, the latter strategy has received more attention. It has been demonstrated that generally, the adsorption of opsonins is favoured onto hydrophobic rather than hydrophilic surface so that hydrophobic molecules are rapidly scavenged. To overcome this major drawback, various encouraging investigations have demonstrated that suitable modifications of the surface properties of drug-loaded colloïdal carriers can avoid or minimize their in vivo rapid recognition and MPS uptake. Thus, the reduction of their clearance rate allows an enhanced localization of these long-circulating colloidal carriers at the target sites. Coating the particle surface with hydrophilic layers such as poloxamers, poloxamines, PEG or incorporating gangliosides (e.g. GM_1) into the lipid bilayer of liposomes represent the different ways of increasing the hydrophilicity of colloidal carrier surface [86,89,90]. Allémann et al. [91] compared the biodistribution of $ZnPcF_{16}$ incorporated in three different vehicles (plain PLA nanoparticles (PLA-NP), CRM and PEG-coated PLA nanoparticles (PEG-PLA-NP)) in EMT-6 mammary tumour-bearing mice. The results showed a significantly different distribution of $ZnPcF_{16}$ in EMT-6 tumour after injection of 0.865 mg kg^{-1} dye loaded in PEG-coated PLA nanoparticles or in plain nanoparticles. In the case of plain nanoparticles, a rapid clearance from the bloodstream was observed and a high concentration of the dye was found in the liver, spleen and lungs. About 64% of injected $ZnPcF_{16}$ was accumulated in these three organs at 24 h p.i., while for CRM and PEG-PLA-NP formulations, the cumulative doses were 56 and 45%, respectively. After 24 h, tumour concentration was 1.20, 2.78 and 3.63 μg/g for PLA-NP, PEG-PLA-NP and CRM, respectively. Maximal tumour levels for the latter formulations were observed at different times, i.e. 4.01 μg/g for PEG-PLA NP at 48 p.i. and 3.63 μg/g for CRM at 24 p.i. Indeed, 24 h p.i. of a dye dose of 5 μmol/kg, PDT results showed 100% of the mice cured treated with PEG-PLA NP as opposed to only 60% with the CRM emulsion [91].

The protecting effect of poloxamers was also evaluated by Lenaerts et al. [65]. They studied the influence of three kinds of poloxamers (238, 403, 407) used to coat the ZnPc loaded nanocapsules on their distribution after intravenous administration to healthy mice. Under these conditions, poloxamer 403 was less effective than the other poloxamers in reducing recognition and uptake by spleen but it provided a good protecting effect against liver uptake. This may be explained by its shorter lateral chains, which provide a thinner coating and a lower apparent hydrophilicity. This point confirms the correlation between the decrease in uptake and the increase in coating layer thickness. In contrast, nanocapsules coated with more hydrophilic substances are able to reduce both liver and spleen uptake. However, increasing hydrophilicity alone is not sufficient if complement activating groups (e.g. hydroxyl groups) are also present [90]. These hydrophilic layers of polymer must generate a steric barrier between the carrier surface and opsonins. It is known that the efficiency of this steric hindrance to repel MPS and to increase the circulation time of colloidal carriers depend on the molecular mass, the surface density and chain length of the polymer [89,92,93].

The long circulating strategy has been described using liposomes to modify the components of the phospholipid bilayers [94]. Oku et al. [95] used BPD-MA encapsulated into glucuronate-modified liposomes. They incorporated a glucuronate derivative, palmityl-D-glucuronide (PGlcUA) in the liposome bilayer (PGlcUA-liposome) to obtain a long-circulation vehicle. A PDT comparative study was performed with three formulations namely, PGlcUA-liposomes, DMSO solubilized BPD-MA, conventional liposomes. Meth A sarcoma-bearing mice were used as animal models. At the dose of 2 mg/kg of BPD-MA, the PGlcUA-liposomes were the most efficient in reducing tumour growth in comparison with the conventional liposomes or DMSO solubilized BPD-MA. No significant side effect was observed (i.e. no weight loss in mice). At higher doses of BPD-MA (6 mg/kg), PDT with PGdlcUA liposomes induced a drastic regression of tumours and complete cure of tumour. Eighty percent of the animals survived more than 180 days without any tumour regrowth, whereas only 20% survived with BPD-MA solubilized in DMSO or conventional BPD-MA liposomes.

5. Active targeting systems

To provide selective delivery of PS to a tumoral tissue, investigators have turned towards the targeted PS approach including conjugates containing a receptor-targeting moiety and PS [22]. Theoretically, this approach has several advantages: (i) high affinity of the binding moiety to the receptor or antigen on the targeted cell surface; (ii) direct and more specific localization with increased efficiency and selectivity; and (iii) lower effective dose of PS. The efficiency of this strategy is governed by several factors such as: (i) the stability of the conjugate in blood circulation and its protection against a possible inactivation during the transport and activation at the target tissue; (ii) the ability of the conjugate to cross the physiological barriers and reach its site of recognition before internalization by endocytosis; and (iii) the nontoxicity, nonimmunogenicity and biodegradability of the conjugate [96].

Lipoprotein-mediated delivery and monoclonal antibodies have been used to reach these goals.

5.1. Lipoprotein-mediated delivery

In recent years, more attention has been focused on the clinical use of plasma lipoproteins following the establishing of a direct relationship between the relative number of lipoprotein receptors in various tumours and the uptake of PS by malignant cells [97–104]. In the lipoprotein class, low-density lipoproteins (LDL) have been suggested as playing an important role for the transport and release of photosensitizing molecules to tumour cells. This can be explained by the fact that tumours are generally characterized by frequently proliferating cells and high permeability. In fact, the expression of LDL receptors on the cell surface cell is regulated by its need for cholesterol. It is known that rapidly proliferating tissues have a high demand for cholesterol for cell membrane synthesis. The cholesterol may either be obtained by de novo synthesis or from uptake of LDL via receptor (Apo B/E receptors) pathway activity and non-receptor-mediated process (passive diffusion) [27,100,105–110]. In addition, many tumour cells have lost control over the normal regulatory mechanisms and express an abnormally high number of LDL-receptors, unlike normal cells [102,105,111,112]. These endogenous entities are composed of: (a) a lipophilic core mainly constituted of cholesterol esters; and (b) a surface monolayer formed of phospholipids and unesterified cholesterol and a recognition moiety in the form of apoprotein B-100 which is the essential element of targeting. Used as drug delivery systems, lipoproteins have the following advantages: (i) due to their natural components, lipoproteins escape recognition by the MPS and thus have a long circulation time in the plasma and biological fluids and they are not immunogenic; (ii) their small size (mean diameter of 10–23 nm) favour diffusion through the vascular membrane; and (iii) their apolar core constitutes an excellent domain for lipophilic drugs, which are thus protected from the blood environment during transport. In addition, the localization of the PS in lipophilic core of lipoproteins does not alter their recognition by cell receptors [105,112]. Several studies have shown that the LDL may be excellent carriers or drug delivery systems and may enhance the efficiency and selectivity of active agents. However, the natural selectivity of these delivery systems depends upon the rate of LDL tumour uptake [101,113]. Hydrophobic PS are spontaneously incorporated into the lipid core lipoproteins without changing the physical or biological properties of the particles (no alteration of the recognition of LDL by LDL receptor). The resulting complex has been found to be effective in the treatment of animal tumours. For instance, Candide et al. [114] have shown that it was possible to incorporate 130 Photofrin® II (P2) molecules into one LDL entity. No significant alteration of the interaction between LDL and its membrane specific receptor in cultured human fibroblasts was observed in spite of a slight increase of the negative charge of LDL.

Several studies have shown that PS mixed non-covalently with LDL before administration led to an increase in photodynamic efficiency in comparison with the administration of PS alone (Table 3). Barel et al. [115] noted that after i.v administration to healthy rabbits, free Hp associated with the three main lipoproteins (VLDL, LDL and HDL) with varying efficiency. After administration of Hp complexed with each type of lipoproteins (by a mixing technique) to tumour-bearing mice, the specific uptake of Hp-LDL complex by MS-2 fibrosarcoma tumour was higher than Hp-HDL, Hp-VLDL or free dye. Tumour/liver ratio at 24 h after intracardiac injection of dye (1 mg/kg) was 2.97 for Hp-LDL complex whereas it was of 0.81 for Hp-HDL, 1.51 for Hp-VLDL or 0.97 for free dye. These findings are in agreement with those of Candide et al. [114]. Indeed, at a dose of 10 μg g/ml, Photofrin® II (P2) delivered by LDL-P2 was about three times more efficient than HDL-P2 and 5-fold more than 250 μg g/ml of

Table 3
Results of tumour accumulation and phototoxicity of PS delivered by lipoproteins

PS	Carriers	Dye dose (mg/kg)	Target	Host	Comments	Ref.
ZnPc	LDL	0.12	MS-2 fibrosarcoma	Mice	Tumour accumulation of dye was 2-fold higher as compared to liposomal formulation.	[118]
BPD-MA	LDL HDL	4–6	M1 rhabdomyosarcoma	Mice	BPD-MA precomplexed with LDL led to a significantly higher tumour cell killing (51%) at 8 h p.i. compared to control.	[116]
BPD-MA	LDL	2	CNV	Rabbit	PDT induced total choroicapillary closure with minimal retinal damage at 3 h p.i.	[117]
BPD-MA	LDL	4	M1 rhabdomyosarcoma	Mice	LDL receptor was responsible for tumour uptake of BPD-LDL both in vitro and in vivo.	[147]
BPD-MA	LDL Ac-LDL Liposome	6 6 6	Artherosclerotic plaques	Rabbit	Preferential accumulation of dye in artherosclerotic plaques when the dye was preassociated with lipoproteins.	[148]
Ce	LDL	4 Nmol	Human retinoblastoma cells	None	Highest phototoxicity was achieved at binding ratio 50:1 (Ce_6:LDL). The uptake was 3- to 4-fold higher than uptake of free dye.	[119]

aqueous P2 solution. These results may be explained by the fact that P2 alone aggregates in aqueous media, which may affect its chemical properties. This may lead to an alteration of dye distribution among serum proteins and may hamper their tumour accumulation. Similar results were observed by Allison et al. [116]. They found that at 3 h p.i., an increased level of BPD-MA was delivered to M-1 tumour when the drug was linked with LDL and HDL. Futhermore, in vivo cytotoxicity assays showed a great efficiency of PDT in comparison with the dye mixed with unfractionated plasma. In contrast, with the irradiation at 125 J/cm^2 at 8 h p.i. of the dye, only LDL and BPD-MA mixtures led to greatly enhanced photodynamic killing compared to plasma control.

Lipoprotein-complexed photosensitizer may also be selectively accumulated in the neovasculature due to an increase in LDL receptors resulting from the rapid proliferation of the endothelium and a facilitated transport across the endothelium of permeable vessels. Schmidt-Erfurth et al. [117] have used BPD-MA complexed with LDL to improve drug delivery to choroidal neovasculature in rabbit. Thus, complete choriocapillary occlusion was obtained using LDL and BPD-MA mixture at a dose of 2 mg/kg without serious damage to neural retina when the irradiation by light at 10 J/cm^2 was performed 3 h p.i., time at which the highest tissue levels were reached.

Reddi et al. [118] have carried out a comparative pharmacokinetic study of ZnPc injected into MS-2 fibrosarcoma-bearing mice after incorporation into DPPC liposomes (ZnPc–DPPC) or after complexation to human LDL (ZnPc–LDL). They found that regardless of the delivery systems used, the elimination of the dye from the serum followed biphasic kinetics: elimination of the majority of dye (~70%) in about 12 h p.i. followed by a slow phase of elimination. Maximal tumour accumulation (0.55 μg g^{-1} tissue at 24 h) using ZnPc–LDL was 2-fold higher as compared with ZnPc–DPPC. The tumour/muscle ratios were higher after 24 h p.i. (i.e. 3.7 and 5.7 for ZnPc–DPPC and ZnPc–LDL, respectively). But these maxima were short lasting because tumour concentration was decreased to ~0.43 μg/g at 45 h p.i. Nevertheless, it appears that with simple mixing of PS with LDL, a redistribution of PS among non tumour-homing plasma proteins may occur. This is certainly due to the unstable interaction between two entities, which limits the selectivity of tumour targeting. To overcome this problem, many authors have proposed chemical binding. Schmidt-Erfurth et al. [119] have covalently linked Chlorin (Ce_6) to LDL by a peptide bond catalysed by a carbodiimide activation. The optimal binding ratio, which provides the maximal cellular uptake efficiency, is 50:1 (Ce_6:LDL). At this optimal ratio, the in vitro uptake of Ce_6:LDL conjugate by fibrosarcoma and retinoblastoma cells was 3- to 4-fold higher than of the dye alone whereas the uptake of Ce_6 and LDL mixed (Ce_6-mix) led to a virtually non-selective uptake of the free dye. After irradiation of light at 10 J cm^{-2}, Ce_6:LDL provides 20% survival of retinoblastoma cells against 80–90% with Ce_6-mix or ~100% with free dye.

Although interesting data have been gathered in vitro on animal models, clinical usefulness of the lipoproteins as delivery systems comes up against some problems. The data obtained in animals are not readily transposable to human because it has been established that the serum protein composition is different between animal species, between individuals of the same species and it is also affected by physiological characteristics of the individual (e.g. metabolic functions), and dietary regime. In fact, large differences were observed between humans and rodents such as rats and mice, which are normally used in experimental PDT studies [103]. These animals present a very low amount of circulating LDL (0.6 mg/ml in mice) as compared with humans (1–2 mg/ml). Allison et al. [116] have recognized that the choice of this model animal for assays using human lipoproteins was not ideal but they have obtained encouraging results. In fact, it has been shown that the murine tissues also express both receptor mediated and receptor-independent uptake of human LDL. However, the results of these investigations in animal models may not reflect the real situation in the human organism and a direct extrapolation of the results to a human model may be complex and limited.

5.2. Antibody-targeted delivery

Antibody (Ab)-based drug delivery is another approach to improve the specificity of PDT and to overcome side effects associated with this therapy. This strategy consists of linking PS with monoclonal antibodies (MoAb) against specific antigens of malignant cells. Theoretically, MoAb hold great promise for facilitating the transport of various agents to suitable cellular targets because of the specificity of MoAb–antigen interactions. It is known that there are characteristic differences between tumour and normal cells, e.g., higher expression of specific antigens and oncoproteins by cancer cells [120,121].

Some in vitro investigations of immunoconjugated PS have led to encouraging results, particularly for small tumours and ascite tumours which are suitable for this type of treatment. It has been established that the linkage of MoAb to molecules including PS and cytotoxic agents was possible without significant loss of either drug activity or MoAb specificity. The results of these investigations have also shown that a lower effective dose of immunoconjugate was necessary to produce higher selective phototoxicity effect over drug or MoAb alone (Table 4).

A preliminary study of the feasibility of photoimmunotherapy as a potential treatment for cancer has been carried out by Mew et al. [122]. They directly attached anti-myosarcoma M1 (anti-M1) to Hp via random peptide bond formation catalysed by carbodiimide reaction. The in vitro phototoxicity assays showed that anti-M1–Hp conju-

Table 4
Tumour uptake and photodynamic activity of PS-MoAb or other specific tumour-seeking molecule conjugates

PS	MoAb carrier	Spacer	Tumour (host)	Comments	Ref.
Hp	Anti-M1	None	M1 rhabdomyo-sarcoma (mice)	Significant tumour growth retardation at lower conjugate dose as opposed to Hp alone.	[122,149]
Hp	CAMAL-1	None	M1 rhabdomyo-sarcoma (cells)	Doses as low as 1.2 ng per 10^6 cells led to significant cell death compared to controls.	[149]
Ce_6	Anti-Leu-1	Dextran	Human T leukaemia (cells)	Chlorin:MoAb ratio was 30:1. Fraction of dead cells was 90% after irradiation at 58 J/cm^2.	[150]
CMA	Anti-Leu-1	PGA	Human T leukaemia (cells)	CMA:MoAb ratio was 36:1 with 70% binding activity and specificity.	[151]
AlSPc	Liposome-791T/36	PVAL	Osteosarcoma (791T) (cells) Colorectal carcinoma (C170) cells)	At 2.5 μg/ml, targeted AlSPc liposomes were less toxic for C170 cells while at 4.25 μg/ml, equal cytotoxicity was observed for both cell types.	[135]
BPD-MA	5E8	PVAL	Human chorionogo-nadotropic hormone (cells)	The phototoxicity of BPD-co-PVA-5E8 was 15-fold higher than free dye and 10-fold higher compared with BPD-co-PVA-T48.	[109]
Ce_6	17.1A	Poly-L-lysine	Liver metastases of colorectal cancer (HT29 cells)	The cationic conjugate delivered four times more Ce_6 to the cells than the anionic conjugate. After incubation with 1 μM Ce_6, illimination with only 3 J cm^{-2} of 666 nm light reduced at 90% the cell colonies for cationic conjugate against 73 and 35% for the anionic conjugate and free Ce_6, respectively.	[128]
Ce_6	17.1A	Poly-L-lysine	Liver metastases of colorectal cancer (mice)	After i.p. injection, the anionic conjugate delivered more than twice Ce_6 to the tumour at 3 h than other species. Tunour:skin ratios were high for all conjugates but not for free dye.	[129]
Ce_6	17.1A	Poly-L-lysine (Polyanionic)	Liver metastases of colorectal cancer (mice)	A high significant reduction in the weight of the tumours was obtained and the median survival were increased from 62.5 to 102 days.	[130]
m-THPC	U36	None	Squamous cell carcinoma (mice)	m-THPC-MoAb U36 was rapidly cleared from the bloodstream and was significantly less effective in vitro PDT, at equimolar doses, than free dye.	[132]
$SnCe_6$(ED)	EGF	Dex HSA	Human breast adeno-carcinoma (cells)	The affinity of EGF for its receptor was considerably diminished when conjugated in EGF-HSA-$SnCe_6$(ED) in contrast to EGF-Dex-$SnCe_6$(ED). The higher intracellular accumulation (5-fold higher) and photocytotoxity on breast adenocarcinoma cells were observed with EGF-HSA-$SnCe_6$(ED) (IC_{50}, 63 nM) at light dose of 27 kJ/m$^{2.}$	[142]
Ce_6	Insulin	BSA	Human hepatoma PLC/PRF/5 cells	The EC_{50} for the conjugate was 100 times lower than that of the free Ce_6 and the photodynamic activity of BSA-insulin-Ce_6 was three to four times higher efficient compared to the free dye.	[145]

gate eliminated 95% T1-tumour cells whereas the equivalent concentration of Hp or MoAb alone produced no effect on tumour growth. In vivo antitumour activity test in tumour-bearing mice indicated that anti-M1–Hp conjugate provided tumour growth retardation at a 10-fold lower concentration (0.268 mg/kg body weight) than the dose of Hp alone inducing the same delay of tumour growth. In another study [122] Hp–CAMAL-1 conjugate against a leukaemia-associated antigen exhibited significant specific killing even at the lowest doses of dye. The killing effectiveness of Hp was enhanced by two orders of magnitude.

The direct attachment of molecules to MoAb is easy to carry out and has the advantage of occurring under very mild conditions. Nevertheless, it has not provided total satisfaction since an increase in the degree of substitution leads to a significant loss of antigenic specificity of the MoAb. The loss of antibody activity is probably due to some modifications in the physico–chemical properties of the MoAb [21]. This effectively limits the molar drug-to-antibody ratios. In addition, binding hydrophobic PS may alter the antibody solubility and restrict the number of PS that can be delivered by one antibody [96]. In addition, this technique does not appear easily reproducible since the conjugation occurred randomly and as proteins contain amino groups and carboxyl groups, both intra- and inter-

molecular cross-linking in the MoAb molecule can occur [123]. To overcome these problems, some researchers have recommended a ''spacer-arm'' in which the PS is coupled to MoAb via polysaccharide or polymeric carriers such as dextran [36], polyglutamic acid (PGA) [123], or PVAL [109,124,125]. The advantage of this indirect procedure is that it conjugates a large number of PS molecules to MoAb. This second strategy was first described by Oseroff et al. [126] where Ce_6 was linked to MoAb against T-cells (anti-Leu-1) using dextran carriers. The ratio of the immunoconjugation was 36 mol PS/mol MoAb and up to 85% of antigen binding activity was retained. The Ce_6 absorption spectrum was not disturbed and the quantum yield of O_2 generated remained the same as with free Ce_6 (~0.7). However, the risk of MoAb-MoAb cross-linking due to the excess of free amino groups on dextran and the possible formation of high aggregates are some drawbacks of this binding method [21]. Thus, Steele et al. [124] proposed a new technique for reliable attachment of Hp to MoAb using modified PVAL as immediate carrier. This procedure was based on the coupling of PS to a modified PVAL by using carbodiimide. The PS-co-PVAL was subsequently loaded onto the MoAb using heterobifunctional linkers. The use of PVAL, a water-soluble carrier, provides favourable binding properties between both entities and a good water solubility of the final conjugate PS–co-PVAL–MoAb. They observed a transient positive immune response at the time of the injection of P815 cells in mice. But, this positive effect of T lymphocytes was rapidly neutralized by the development of T suppressor (Ts) cells. So, they tried to selectively eliminate Ts cells using hematoporphyrin linked to B16G (Hp-B16G) which is able to selectively react with Ts cells. The results of this suppressor deletion therapy showed that a significant number of mice treated with Hp-B16G presented tumour regression, slowed tumour growth or even total regression. The injection of Hp alone or a mixture of Hp and B16G control animals showed no significant difference in the growth rate or in survival times in comparison to animals treated with phosphate buffered saline. Jiang et al. [125] used this reproducible and quantifiable technique to conjugate specific MoAb (T48) for human chorionogonadotropic hormone with BPD-MA using modified PVAL as immediate carrier. In a subsequent study [109], they also tested the same dye linked to MoAb (5E8), which reacts with a cell surface glycoprotein, associated with human squamous cell carcinoma of the lung. In vitro, BPD-co-PVAL-5E8 was 15-fold more phototoxic than the dye alone and 10-fold more effective in comparison with a non-specific control conjugate (BPD-co-PVAL-T48). Under their conditions, they have also found that the internalization of BPD via either MoAb conjugate increased significantly (10-fold) photodynamic killing of cells probably due to a rapid delivery of cytotoxic drug by the conjugate.

Recently, a method of preparing photoimmunoconjugate using poly-L-lysine as a linker to attach several molecules of Ce_6 and which enables the obtention of polycationic or polyanionic charges was reported [72,127,128]. Both charged immunoconjugates preserved most of the immunoreactivity and showed more selective uptake and phototoxicity towards target HT29 human colorectal cancer cells than given by the free Ce_6 [128]. In a previous study, it was reported that both size and charge of the conjugate had a significant influence on the biodistribution of the dye [73]. In vitro, it was noted that the cationic conjugate delivered four times more Ce_6 to the cells than its corresponding anionic conjugate. However, in vivo, the polyanionic conjugate was found to give much better accumulation of the dye in the tumour than cationic conjugate after i.v. injection, and it conferred a distinct improvement in tumour uptake compared to surrounding normal tissue [129]. Cationic charges led to a very high uptake in the lungs and relatively low levels in blood and other organs. After intraperitoneal (i.p.) injection, the higher amount of the dye reaching the tumour was observed with cationic conjugate [127]. The conclusion drawn from these studies was that polyanionic conjugates perform better after i.v. injection, whereas polyanionic ones were better for injections in cavities. The PDT of hepatic metastases of colorectal cancer in an orthotopic murine xenograft using polyanionic conjugate showed promising results in destroying liver tumours [130] but the optimal parameters enhancing PDT selectivity are not yet established. Rapid elimination of the PS-MoAb conjugate from the bloodstream compared to the unconjugate species was often described. Vrouenraets et al. [131] have shown that after the i.v. injection of m-THPC coupling to MoAb U36 against head and neck squamous cell carcinoma-bearing nude mice (m-THPC:MoAb U36), the clearance was more pronounced for the conjugates with the higher m-THPC:MoAb U36 ratio. m-THPC:MoAb U36 was effective in vitro PDT but significantly less effective, at equimolar doses, than the free m-THPC. The same conclusions were subsequently drawn in the same tumour-bearing nude mice using TrisMPyP-ϕCO_2H as hydrophilic photosensitizer [132].

Another approach to increase the drug loading on the MoAb molecule is to use loaded colloidal carrier systems coated with MoAb. It has been shown that specific delivery of drugs to the target cells is far more efficient by using colloidal carrier coated with MoAb rather than without antibody [133]. In addition, it is possible to coat the surface of these carrier systems with a large number of antibodies (e.g. an average of 935 antibody molecules per liposome). MoAb may be linked to the colloidal carrier, e.g. liposome and polymer particle surface by a non-covalent link (specific adsorption by simple incubation) or by covalent interactions [123,134]. Morgan et al. [135] have encapsulated sulfonated aluminium phthalocyanine (AlSPc) in liposomes coated with MoAb 791 T/36 (using a heterobifunctional reaction). Under their conditions, the in vitro photodynamic assays against two cell lines (osteosar-

coma 719T and colorectoral carcinoma C170) showed an instantaneous production of free radicals and a highly specific cell killing by AlSPc-liposome-MoAb 791 T/36, in comparison to a control cell line not bearing the antigen. Indeed, this cytotoxic effect depends on the concentration of the dye, the density of antigens and the time of exposure to activating red light. However, only a limited number of preclinical studies report successful targeting of MoAb-coated particles in vivo because they were taken up by MPS. This can be significantly reduced by appropriate coating agents (see Section 4) and by the use of $F(ab')_2$ fragments instead of the whole MoAb [136]. The intracavity administration of immunoconjugate in the target sites is a possibility to reduce MPS uptake [137–139]. In addition, this type of administration, the aim of which is regional treatment, has the potential advantage of exposing the target tumour a high immunoconjugate concentration [138].

In spite of the numerous encouraging in vitro results, many problems must be overcome before clinical trials can be envisioned. In vivo efficacy of PS-immunoconjugates depends on several factors which may influence tumour access, tumour localization of the targeted MoAb, its biodistribution in the human organism [140] and the density of tumour antigens on the cell surface as well as on the heterogeneous expression of the antigens both within and between tumours [141]. In fact, the tumour cell population is not a static entity. The perpetual changes in the antigen profile of tumour cells lead to the loss of specific receptors, down-regulation or concealing antigens. This low density of antigens severely hampers the effective localization of the MoAb conjugates and is one of the main causes of immunoconjugate drug therapy failure. To overcome this major obstacle a number of authors [121] have suggested the use of a cocktail of MoAb directed against different antigens in order to achieve maximum cell kill.

Another problem lies in the fact that generally, the majority of antibodies used in clinical investigations are MoAb of murine origin, consequently, the repetitive administration of non-human derived immunoconjugate may induce anti-idiotype antibody reaction [121,141]. Murine MoAb conjugates are recognized as foreign and neutralized by human antibodies. This immune response in humans against the murine MoAb leads to an increase of the conjugate clearance from blood circulation resulting in poor localization of PS-MoAb in the tumour. It seems difficult to fully overcome this problem of immunogenicity of which the incidence and frequency vary with tumour types. In order to alleviate the immunogenicity, various approaches have been explored [120]. The use of human antibodies as the targeting moiety and CDR-grafted, associated with a technique for reducing the intensity of the immune response (e.g. use of immunosuppressive agents such as cyclosporine A) have been suggested.

Enhancement of photodynamic selective effects and a decrease in the required dose of a PS administered may be also accomplished by using specific tumour-seeking molecules such as growth factors (e.g. epidermal growth factor (EGF)) [142,143], specific proteins (e.g. transferrin) [144] or hormones (e.g. insulin) [145] as an alternative to MoAb. These ligands can be either directly linked to the PS or via a hydrophilic spacer such as albumin, dextran (Dex) and PVAL. However, Gijsens et al. [142] have demonstrated that the intracellular accumulation of the conjugate is extensively affected by the nature of ligand and/or the conjugate chemistry. Indeed, the authors described the photodynamic activity of Sn(IV)chlorin e_6 monoethylenediamine ($SnCe_6$(ED)) conjugated to EGF via two water-soluble carriers, Dex (EGF–Dex–$SnCe_6$(ED)) or human serum albumin (HSA) (EGF–HSA–$SnCe_6$(ED)). The results showed that the affinity of EGF for its receptor was considerably reduced when conjugated with HSA in contrast to Dex. Consequently, the high intracellular accumulation (5-fold higher) and the photocytotoxicity on breast adenocarcinoma cells were achieved with EGF–HSA–$SnCe_6$(ED) (IC_{50}, 63 nM) at light dose of 27 kJ/m^2. Akhlynina et al. [145] have also demonstrated that the best photodynamic activity against human hepatoma PLC/PRF/5 cells was also achieved using a serum albumin conjugate BSA-insulin-Ce_6 (1:13:16). The IC_{50} obtained with this conjugate was about 100 times lower than that of the free PS. Lower doses of irradiation were also necessary to activate the conjugate (26 kJ/m^2) compared to free dye (95 kJ/m^2) indicating that the conjugate was about three to four times more efficient in terms of photodynamic activity. Despite these interesting results, the use of insuline-PS conjugate in cancer therapy is certainly limited since certain hepatoma cell lines do not possess a great number of insulin receptors.

6. Conclusions

Today, PDT is at an exciting stage of development providing considerable research opportunities. For example, there have been few clinical studies that have evaluated the effect of different delivery systems on the relative efficiency of the delivering the same PS. The successful clinical use of PDT requires the selection of the most appropriate PS dose and a suitable PS delivery system and the optimal time of the activation with light after PS administration. Besides the physicochemical properties of the PS, the preferential accumulation in target tissue, dark toxicity, phototoxicity, suitable intravenous injectable formulations are the main factors that govern the development of an effective PS. In vitro or in vivo results of investigations into the development of PS drug delivery systems have demonstrated that the use of vehicles does not simply improve the parenteral administration of the PS but may also influence dye uptake by the diseased tissues. Most studies performed in experimental animal species

bearing different tumour models have shown that the delivery systems provide a heightened tumour accumulation of PS thus enhancing their therapeutic potential. So, the choice of the suitable delivery system for appropriate PS and tumour type turns out to be extremely important since the mode of delivery of PS affects the biodistribution and pharmacokinetics including transfer to serum proteins [20].

7. Abbreviations

AcLDL	acetyl low density lipoproteins
AlClPc	aluminium chloride phthalocyanine
AlSPc	sulfonated aluminium phthalocyanine
Anti-M1	anti-myosarcoma M1
BPD-MA	benzoporphyrin derivative monoacid ring A
γ-CD	gamma-cyclodextrine
Ce_6	chlorin e_6
Ce_6-MS	chlorin e_6 microspheres
C8KC	ketochlorin
CMA	chlorin e_6 monoethylenediamine monoamide
CNV	choroid neovascularization
CRM	cremophor-EL
DMSO	dimethylsulfoxide
DPPC	dipalmitolyphosphatidylcholine
EGF	epidermal growth factor
Ge(IV)Pc	germanium phthalocyanine
γ-CD	gamma cyclodextrine
Dex	dextran
DMPC	L-∝-dimyristoyl-phosphatidylcholine
HpD	hematoporphyrin derivative
Hp	hematoporphyrin
HDL	high density lipoproteins
HPMA	*N*-(2-hydroxypropyl)methacrylamide
HSA	human serum albumin
i.p.	intraperitoneal
i.v.	intravenous
LDL	low density lipoproteins
MCe_6	mesochlorin e_6 monoethylenediamine
MF	monomeric fraction
MGH-U1	human bladder carcinoma cell
MoAb	monoclonal antibodies
MOL	molecusol
MPS	mononuclunear phagocytic system
m-THPC	meta-tetra(hydroxyphenyl)chlorin
NP	nanoparticles
NPc	naphthalocyanine
OOPS	1,2-dioleoylphosphatidylserine
P2	Photofrin® II
PBS	phosphate buffered saline
Pc	phthalocyanine
PEBCA	poly(ethylbutylcyanoacrylate)
PEG	polyethylene glycol
p.i.	post-injection
PIBCA	poly(isobutylcyanoacrylate)
PDT	photodynamic therapy
PGlcUA	palmityl-D-glucuronide
PLA	poly(D,L-lactic acid)
POPC	1-palmitoyl-2-oleoylphosphatidylcholine
PS	photosensitizers
PVAL	poly(vinyl alcohol)
SiPc	bis(methyloxyethyleneoxy)silicon-phthalocyanine
$SnCe_6$(ED)	Sn(IV)chlorin e_6 monoethylenediamine
$SnET_2$	Sn(IV)-etipurpurin dichloride
TM cells	phagocytic trabecular meshwork cells
Ts	T suppressor cells
VLDL	very low density lipoproteins
ZnPc	zinc phthalocyanine
$ZnPcS_2$	disulfonated zinc phthalocyanine
$ZnPcS_4$	tetrasulfonated zinc phthalocyanine
$ZnPcF_{16}$	hexadecafluorinated zinc phthalocyanine

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