Phthalocyanines Covalently Bound to Biomolecules for a Targeted Photodynamic Therapy

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Abstract: Photodynamic therapy (PDT) is a relatively new cytotoxic treatment, predominantly used in anticancer approaches, that depends on the retention of photosensitizers in tumor and their activation after light exposure. This technology is based on the light excitation of a photosensitizer which induce very localized oxidative damages within the cells by formation of highly reactive oxygen species, the most important being singlet oxygen. Many photo-activable molecules have been synthesized such as porphyrins, chlorins and more recently phthalocyanines which present a strong light absorption at wavelengths around 670 nm and are therefore well-adapted to the optical window required for PDT application.

However, the lack of selective accumulation of these photo-activable molecules within tumor tissue is a major problem in PDT, and one research area of importance is developing targeted photosensitizers. Indeed, targeted photodynamic therapy offers the advantage to enhance photodynamic efficiency by directly targeting diseased cells or tissues.

Many attempts have been made to either increase the uptake of the dye by the target cells and tissues or to improve subcellular localization so as to deliver the dye to photosensitive sites within the cells. The aim of this review is to present the actual state of the development of phthalocyanines covalently conjugated with biomolecules that possess a marked selectivity towards cancer cells; for some of them their photophysical properties and photodynamic activity will be presented.

Keywords: Phthalocyanines, biomolecules, photodynamic therapy, targeted delivery.

1. INTRODUCTION

Photodynamic therapy (PDT) is now well established as a clinical treatment modality for various diseases, including cancer. It involves a photosensitizer, light and molecular oxygen, whose combined action results in the formation of singlet oxygen, which is thought to be the main mediator of cellular death induced by PDT.

PDT is being developed as a treatment for cancer of the oesophagus [1], bronchi [2, 3] and bladder [4, 5], as well as for other non-oncological applications, such as the treatment of age-related macular degeneration [6]. PDT is also used as a successful non-invasive therapeutic modality for treating cutaneous neoplasm [7, 8]. A limitation of PDT is that it cannot cure advanced disseminated disease because irradiation of the whole body with appropriate doses is not possible. Nevertheless, for advanced disease, PDT can enhance quality of life and lengthen survival. For early or localized disease, PDT can be a selective and curative therapy with many potential advantages over available alternatives.

The successful realization of any PDT concept crucially depends on the nature of the photosensitizer [9], classically defined as a chemical entity, which, upon absorption of light, induces a chemical or physical alteration of another chemical entity. Early preparations of photosensitizers for PDT were based on a complex mixture of porphyrins called haematoporphyrin derivative (HpD). Today, Photofrin®, a purified fraction of HpD derivative, is the most commonly used photosensitizer and the only drug approved by the Food and Drug Administration for the treatment of superficial bladder cancer in Canada and early lung and advanced oesophageal cancers in the Netherlands and Japan. Despite its effectiveness photofrin sodium has several drawbacks. The drug induces protracted skin photosensitivity, and the initial selectivity for the tumor tissue is low [10, 11]. With the exception of photofrin sodium, the only other PDT compound currently approved for systemic use in cancer treatment is temoporfin. Temoporfin (meta-tetra(hydroxyphenyl)chlorin, m-THPC, Foscan®) is effective for the palliative treatment of head and neck cancers and it was approved in Europe for this indication in 2001. Nevertheless, like photofrin sodium, temoporfin is also associated with a pronounced skin photosensitivity and exhibits a weak selectivity between tumor and healthy tissue [12]. The benzoporphyrin derivative verteporfin (Visudyne®) was also recently approved, but it has only been developed for the treatment of age-related macular degeneration, and not indicated for cancer [13]. Concerning photosensitizers for topical application to treat skin lesions, protoporphyrin IX (PpIX) can be produced in nucleated cells after topical application of either aminolevulinic acid (ALA) or methyl aminolevulinate (mALA) to the site of a skin cancer or precancerous lesion. This finding has led to approval of ALA in the USA, and of mALA in Europe [14].

The limiting factor of all those photo-activable molecules is their low absorbance in the optical window for photosensitizer excitation, which reduces their efficiency in terms of singlet oxygen production. Of the major proteins of blood that absorb light, the most important quantitatively is haemoglobin, necessitating illumination of tissues at wavelengths higher than 600 nm to ensure a good penetration of light. At wavelengths higher than 850-900 nm, the photons may not have sufficient energy to participate to photochemical reactions. Therefore, the wavelength range between 600 and 800 nm has been determined as the « therapeutic window » for clinical PDT. To increase the absorbance in this wavelength area, porphyrin isomers such as the porphycenes [15-17], expanded porphyrins [18] such as the texaphyrins [19, 20], and phthalocyanines [21] have been developed and are currently tested for PDT applications.

Phthalocyanines (Pcs) are among the most promising second-generation photosensitizers. Since their discovery in 1907 by Brown and Tcheriac during the synthesis of p-cyanobenzamide from phthalimide and phthalic acid [22], numerous information concerning their synthesis,
photochemical and photophysical properties have emerged [23]. They are now prepared and used industrially as dyes and coloring agents and many reports and patents have been published concerning their application as chemical sensors, optical storage devices, conducting polymers but also as catalysts in organic synthesis [23].

Furthermore, their photophysical properties are of the utmost interest because these azaporphyrins present very strong absorption at longer wavelengths than porphyrins, with a very strong absorption peak in the far-red region of the visible spectra (λ_{max} ≈ 670 nm) where tissue penetration by visible light is improved. This absorption can also be fine-tuned through the addition of substituents to the periphery of the macrocycle, or through the nature and the coordination mode of the chelated metal in the central cavity. According to all these photophysical properties, Pcs are potentially interesting photosensitizing agents for PDT [9], and some of them have already been submitted to clinical trials. Thus, a hydroxy SiPc bearing a long-chain amino as axial ligand (HOSiPcOSi(CH₃)₂(CH₂)₃N(CH₃)₂) has been proposed for clinical trials for the treatment of neoplasms and has been investigated for the sterilization of blood components [12, 24]. In Switzerland, a liposomal preparation of ZnPc has been in early Phase I/II clinical trials for the treatment of squamous cell carcinoma of the upper digestive tract [21, 25]. A mixture of aluminium sulphonated phthalocyanines (Photosense®) has also been used widely in Ukraine for treatment of different cancers [26]. A review from Lukyanets has been published concerning the success of clinical studies using Pcs as photosensitizers in this country [27].

Another limitation of the conventional PDT approach for cancer treatment is usually the lack of selectivity of photosensitizers in terms of cellular localization and cytotoxicity. The ideal drug delivery system should enable the selective accumulation of the photosensitizer within the diseased tissue and the delivery of therapeutic concentrations of photosensitizer to the target site with little or no uptake by non-target cells. Unfortunately, the majority of the photosensitizers are taken up non-selectively by all cell types and consequently they can accumulate in tumor but also in normal cells. The accumulation of a photosensitizer in neoplastic tissue relative to normal tissue depends on the photosensitizer molecule, the normal tissue being considered and the animal tumor model being chosen. The reason for the preferential accumulation in tumor tissue compared with certain normal tissues not belonging to the reticulo-endothelial system is not clearly understood. It may be a result of the greater proliferative rates of neoplastic cells, poorer lymphatic drainage, leaky vasculature, or some specific interaction between the photosensitizer and marker molecules on neoplastic cells. Other factors, such as the secretion of vascular endothelial growth factors, may be crucial in photosensitizers accumulation in tumor tissue [28]. Observations suggested a possible specific interaction of the photosensitizers with tumor vasculature [29]; however, there is also evidence that at longer times after injection of the photosensitizer, the treatment effect becomes distinctly less vascular [30]. One suggested specific interaction has been the low-density LDL receptor - photosensitizer interaction leading to increased photosensitizer concentrations in neoplastic tissue. It is suggested that LDL receptors on tumor cells and on tumor vasculature endothelial cells play a key role in the uptake of photosensitizers, a role that may be direct or receptor mediated. We can notice that to date most first and second-generation photosensitizers studied in the literature display only a slight preference for malignant cells. Specificity for target tissues is poor and arises widely from nonspecific passive uptake modulated by metabolic

Fig. (1). Functionalized zinc phthalocyanines coupled with four glucose moieties.
activity of cancer cells, as demonstrated by the strong correlation between hydrophobicity and in vitro activity [31] as well as extracellular tumor characteristic such as inadequate lymphatic drainage [32], anomalous acid-base status [33], surface charge [34] and density of low-density lipoprotein receptors [35].

A valuable strategy to enable the dose of photosensitizer administered to patients to be reduced and hence minimize the harmful side effects of PDT, is to increase the oncotropicity, that is to say the affinity of the photosensitizer to cancer cells, through targeted strategy [36, 37]. Of course, the carrier must be able to incorporate the photosensitizer without loss of alteration of its activity.

This review deals with phthalocyanines covalently bound to different biomolecules (sugars, steroid hormones, amino acids, proteins...), via a direct coupling or using linkers/spacers. The coupling of a vector can either i) modulate the amphiphilicity and enhance the solubility of these compounds in biological media and prevent self-aggregation (passive targeting), or ii) promote cellular recognition (active targeting), with the aim to increase the biological efficiency. Several of these targeting strategies offer the advantage of transporting the photosensitizer across the cellular plasma membrane, resulting in intracellular accumulation of the phthalocyanine, which may allow for targeting photosensitive intracellular sites, and thus improve photodynamic efficiency.

2. GLYCOCONJUGATED PHTHALOCYANINES

The combination of saccharides with non-natural organic compounds is an important area of research and in particular their conjugation to porphyrins macrocycles has been widely studied for use in PDT [38, 39]. To the best of our knowledge, glucose is the oldest example of a biomolecule covalently conjugated to a phthalocyanine [40]. Originally, the goal of Maillard et al. was to link four glucose moities onto a Zinc(II)phthalocyanine core (ΦΔZnPc = 0.55 in DMF ([41])) in order to prevent its aggregation and to increase its water solubility. Only the synthesis and preliminary characterization data of 1 (Fig. 1) were reported [40].

Recently, in order to modulate the hydrophilic/hydrophobic balance of the photosensitizer, to increase its water solubility and to improve the membrane interaction, Alvarez-Mico et al.

![Scheme 1](image-url). Preparation of galactose-containing silicon(IV) phthalocyanines.
have achieved the first anomeric glycoconjugation to a phthalocyanine (Fig. 1), compounds 2 [42]. They chose glucose as saccharide source among all possible carbohydrates. Cancer cells have in general a high requirement for glucose due to their high metabolic rate and over-express glucose transporters. As expected, compounds 2 (Fig. 1) displayed a very high solubility in water and are potentially attractive candidates for biological tests.

Lee et al. described the preparation and in vitro photodynamic activity of Silicon(IV)-phthalocyanines conjugated to galactose via axial coordination, with the aim to increase the hydrophilicity and to inhibit the aggregation of these compounds, but also, as previously described, to enhance the cellular uptake. By nucleophilic substitution on silicon(IV) phthalocyanine dichloride, four new compounds were prepared in moderate yields, either with two galactose moities (compound 3), or with one galactose and one alcoxy substituent (compounds 4) in order to modulate their amphiphilicity (Scheme 1) [43].

The four compounds were highly soluble in most organic solvents and remained essentially non-aggregated in solution. These compounds exhibited a high photodynamic activity against HepG2 human hepatocellular carcinoma, which can be attributed to the improvement of cellular uptake and to their efficiency to generate singlet oxygen. The singlet oxygen quantum yields were 0.94, 0.79, 0.82 and 0.88 in DMF. Compared to others Silicon(IV) phthalocyanines conjugated via axial coordination with ligands such as polyethylene glycol (ΦΔ = 0.16 – 0.52) or 1,3-bis(dimethylamino)-2-propoxy (ΦΔ = 0.25-0.82) described by Ng et al. [44, 45], these quantum yields of singlet oxygen are quite high. Furthermore, they did not display any dark toxicity at concentrations below 8 µM. Nevertheless, it was also observed that the photodynamic activity decreased with increasing length of the alcoxy chain [43].

A last example of glycoconjugated phthalocyanines recently described by Ribeiro et al. concerns the synthesis of the first phthalocyanine-β-cyclodextrine dyads (Fig. 2) [46].

Fig. (2). Functionalized zinc phthalocyanines with α-β-cyclodextrine.

Fig. (3). Functionalized zinc phthalocyanines with amino acids.
The β-cyclodextrin moiety, which is a cyclic glucose heptamer, imparted a great solubility of the adduct in water owing to the presence of hydrophilic hydroxy groups on the outside of the cavity, and promoted a good amphiphilicity character to the compounds. Dyads 5 presented good UV-Visible absorption properties but in aqueous solution, both monomeric and oligomeric species coexisted, as evidenced by UV-Vis spectra analysis. Nevertheless, this compounds family is of interest as water-soluble.

3. PHTHALOCYANINES CONJUGATED WITH AMINOACIDS OR PEPTIDES

In order to modulate the hydrophilic/hydrophobic balance, hydrophobic phthalocyanines have been coupled with one or several amino acids. Gu et al. followed by Dong et al. have prepared the first water-soluble aminoacid modified phthalocyanine by anchorage of a glycine residu onto Zn(II)Pc [47, 48]. Drechsler et al. increased water solubility of the compounds by coupling an aminoacid in the periphery of the phthalocyanines macrocycles (Fig. 3) [49].

As expected, the two compounds 6 exhibited good or excellent water solubility and presented a weak tendency to form aggregates in solution. Nevertheless, photocytotoxicity towards T47D human mammary carcinoma cells was investigated and the results showed that the tetraamino acids photosensitizers had a poor efficiency and the serine derivative exhibited even a dark toxicity.

The occurrence of apoptosis as a mechanism of PDT-mediated cell death has been previously demonstrated both in vitro [50, 51] and in vivo [52]. Phthalocyanine-mediated PDT has been shown to induce apoptosis and G0/G1 cell cycle arrest in A431 human epidermoid carcinoma cells [53]. Recently, Haywood-Small et al. investigated photodynamic cell cycle deregulation, apoptosis and cell death with different zinc phthalocyanines derivatives in SiHa human cervical carcinoma cells [54]. They compared the efficiency of the zinc phthalocyanine tetra-sulphonic acid (TSPc) with derivatives 7 containing amino acids with increasing alkyl chain lengths (Fig. 4). These conjugates were more hydrophobic than TSPc, but maintained their water solubility. The aminocaproic acid derivative was determined to be the most effective photosensitizer. No photophysical data concerning conjugated phthalocyanines are given in this paper (Φν TSPc = 0.48 in DMF (41)). Photocytotoxicity of the derivatives was of the same order of magnitude as for TSPc but with an overall trend of increased phototoxicity with increasing amino acid chain length. These functionalized phthalocyanines with amino acids induced cell death and caused a G0/G1 cell cycle arrest in a time and dose-dependent manner during PDT. In vivo tests in rodent model systems look promising.

By using solid phase organic synthesis strategies, it also appears possible to bind a functionalized photosensitizer to a pre-assembled side chain protected peptide attached to a solid phase, allowing for a specific coupling of the photosensitizer to the N-terminal part of peptide with no disturbance of the structure. This strategy, which has been largely applied on a large number of photosensitizers [55], has been applied only once on phthalocyanines in one general patent. The approach concerned the synthesis of phthalocyanine analogues bearing a specific reactive group able to link them to a biomolecule through covalent bonds for use in PDT and in vivo diagnosis. Unfortunately neither chemical structures nor photophysical and biological properties were described and only the photoinactivation of C. albicans was very briefly presented [56].

4. PHTHALOCYANINES CONJUGATED WITH PROTEINS

4.1. Serum Proteins

Upon administration in the blood stream, most drugs associate to various serum proteins, including albumin and both high and low density lipoproteins (LDLs), via low non-covalent interactions (H-bonding, van der Waals forces, hydrophobic interactions, etc...). It is expected that a covalent pre-association of a photosensitizer with these carriers could improve its photodynamic action and could enhance its intracellular accumulation via receptor-mediated endocytosis [37].

4.1.1. Albumin

Being the most abundant serum protein in the human blood, with a concentration ten times higher than the total concentration of all proteins, albumin is of high interest. The increase of the metabolism and the proliferation of cancer cells induce a strong and fast serum albumin turnover in tumors. Albumin is able to link, covalently or reversibly, with many endogenous and exogenous compounds, improving the tumor selectivity. Thus non-covalent conjugation of albumins to phthalocyanines has been widely studied [37, 57-60]. It is also known that physically altered albumin is targeted by scavenger receptors, over-expressed on macrophages, which constitute

![Fig. (4). Zinc Pc tetra sulfonic acid with amino acid substituents of varying alkyl chain and degree of branching.](image-url)
over 50% of the tumor cells in several cancers. These scavenger receptors are able to bind a wide range of different compounds, including maleylated Bovine Serum Albumin (mal-BSA), and carrier them to cellular compartments. Some studies have been undertaken to covalently bind various photosensitizers to altered albumin in order to improve photodynamic efficiency [37, 59].

However, hydrophilic photosensitizers do not bind to lipoproteins and thus are not susceptible to natural target to macrophages. To overcome this drawback, a water-soluble tetralsulfonated aluminum phthalocyanine (AlPcS₄) was coupled to mal–BSA in a 9:1 molar ratio via one or two sulfonamidemethanoic-amide spacer chains (Fig. 5), followed by treatment with maleic anhydride to yield the corresponding mal–BSA–phthalocyanine dyad [61]. The authors showed that the relative rate of L-tryptophan to yield HPPI hydroperoxide isomers indicative of singlet oxygen production is divided by 8 comparing the free Pc and the conjugated BSA-Pc.

In vitro experiments were performed in J774 cells of macrophage origin and nonphagocytic EMT-6 cells. It has been shown that covalent conjugates of native or maleylated albumin with sulfophthalocyanines are effectively taken up by cells of macrophage lineage through the scavenger receptor and are able to induce significant cell photoinactivation. Competition studies of the conjugates with ¹²⁵I-mal–BSA showed that coupling of AlPcS₄ to BSA resulted in recognition of the conjugate by the scavenger receptor, whereas coupling to mal–BSA further enhanced its binding affinity. For the authors, photoaffinity for the scavenger receptor was related to overall charge of the protein. Actually the conjugates’ phototoxicity for J774 cells was consistent with their relative affinity (related to the overall negative charge of the protein). For EMT-6 cells, conjugates were less photocytotoxic than the non conjugated AlPcS₄. The activities in both cell lines of all conjugated AlPcS₄ preparations were however lower than that of the free parent disulfonated aluminum Pc (AlPcS₂₃), probably due to an aggregation of the Pc within the BSA pocket, as already observed for the non-covalent mal–BSA–phthalocyanines conjugates [58].

4.1.2. Low-Density Lipoproteins (LDLs)

LDLs are naturally occurring nanoparticles, constituted of about 1500 esterified cholesterol molecules surrounded by a shell of phospholipids and unesterified cholesterol. The principal role of LDLs is to carry cholesterol and other lipids in the blood to various cells. As cholesterol is a key component of plasma membranes, cholesterol is absolutely essential for the cells’ growth and viability. By consequence, it appears natural that tumor cells, in increased proliferation, over-express the LDL receptor through endocytotic processes. This makes LDLs attractive vehicles for drug targeting. Furthermore, another advantage of LDLs in terms of PDT applications is that their light-oxidation during PDT irradiation leads to the formation of highly oxidized cytotoxic species, which enhances the efficiency of PDT treatment. The photosensitization of LDL could trigger an oxidation chain in the lipoprotein particle, producing cytotoxic and atherogenic oxidized LDL particles, which are no longer recognized by the apo B/E receptor of the cells, but are internalized by macrophages in the intima space of arterial walls, giving rise to foam cells and fatty streaks, the initial pathophysiologic lesion of atherosclerosis.

The interactions between LDLs and incorporated Pcs have been widely studied [37]. In order to enhance this incorporation, AlPcS₄ was covalently bound to the protein lysine amine residue of the LDL via two sulfonamidemethanoic-amide spacer chains (Fig. 5), compound 9) [62], using the carbodiimide method [61]. The photocytotoxic activity of the adduct LDL-Pc was measured against EMT-6 and A-549 cell lines. Unfortunately, the covalent label of the protein portion did not enhance the photodynamic efficiency with marginal cytotoxic effects (the non-conjugated adduct being completely inactive), which, according to the authors, is probably due to a reduced receptor recognition of the LDL-Pc conjugate induced by the covalent coupling [37] and to an increased aggregation, as already supposed in related systems [61, 63], leading to a decrease in the singlet oxygen formation. Allen et al. [63] coupled tetralsulfonated aluminum phthalocyanine to adenovirus type 2 capsid proteins including the hexon, the penton base and the fiber to enhance their target selectivity. AlPcS₄ was covalently coupled to the various capsid proteins via one or two caproic acid spacer chains. The photooxidation of L-tryptophan to yield HPPI hydroperoxide isomers indicative of singlet oxygen production is divided between 3 and 10 times when the Pc is bound to a protein subunit relative to AlPcS₄ (Φ₄ = 15% in DMF ([41]).

4.2. Phthalocyanines Conjugated with Epidermal Growth Factor (EGF)

Because a high EGF receptor expression frequently accompanies several tumor types such as squamous carcinomas, its natural ligand EGF was an attractive candidate for the conception of a targeting strategy. One binding to its receptor, EGF is internalized in the cell through receptor-mediated endocytosis, enabling the intracellular accumulation of photosensitizers [64].

Based on this concept, EGF and also VEGF (Vascular Endothelial Growth Factor) receptors provide an interesting
approach for selective and efficient photosensitizer delivery to tumor vessels [65].

To the best of our knowledge, only one article has been reported in the literature concerning the coupling of EGF with Pcs [66]. Lutsenko et al. have conjugated EGF with aluminium (EGF-Pc(Al)) and cobalt disulfonated phthalocyanines (EGF-Pc(Co)), the EGF-Pc ratio being 1:1. The study compared first their photocytotoxic activities with those of non-conjugated Pcs for the human breast carcinoma cell line MCF-7. Two different types of activation mechanisms have been used, photoactivation for EGF-Pc(Al) and activation by ascorbic acid for EGF-Pc(Co). Indeed, the photocytotoxicity of Pcs is not limited only to the generation of $^{1}\text{O}_2$ (a type II energy transfer reaction). Photoinduced electron abstraction from appropriate biomaterial (a type I electron transfer reaction) could give one-electron oxidized primary radicals which can provide the precursors of oxidative damage in Pc photosensitization. It is known that complexation of Pcs with open shell or paramagnetic metal ion such as Co$^{2+}$ gives dyes with shortened triplet lifetime which renders the dye photonactive [67]. Anyway, Co(II)Pcs catalyse the formation of reactive oxygen species such as superoxide radical ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$) and hydroxyl radical (OH) from molecular oxygen and reducing molecules.

EGF-Pcs exhibited a much higher photoactivity than their non-conjugated analogs and the photocytotoxicity of EGF-Pc(Co) conjugate was 4.5 times higher than EGF-Pc(Al). The antitumoral activity of the EGF-Pc-(Co) conjugate and of free Pc(Co) was also investigated in vivo on the growth of solid tumors (murine melanoma cell line B16) implanted in C57B1/6 mice. The administration of the conjugate EGF-Pc-(Co) strongly increased mean life spans and mean survival time of animals bearing tumors. Free Pc(Co) had practically no effect on these parameters [66]. The maximal therapeutic effect needs to be optimized in relation with different schedules for the drug administration.

4.3. Adenoviruses and Adenoviral Proteins

To increase the selectivity of Pcs, Allen et al. have evaluated the use of viral proteins to deliver the photosensitizer to the selected tissue [63]. Adenoviruses (Ads) cause illnesses such as gastroenteritis, conjunctivitis and respiratory diseases. They have received a great deal of attention as gene therapy vectors, owing to the facility and the safety to manipulate and to stock them. It has also been shown that adenoviruses can infect several cells and tissue types and gain access into a cell via receptor-mediated endocytosis. Adenoviruses efficiently break the endosomes upon infection in the cell and therefore, it was anticipated that Ad-PS dyads would target the cell nucleus more quickly than free PS. This targeting of the nuclear cell resulted in a 2.5 fold increase of the photodynamic activity of PS in comparison with a cytoplasmic localisation [63].

The $\alpha_\beta_\beta_\beta$-integrin, a heterodimeric transmembrane glycoprotein receptor, is also over-expressed in many tumor cells, such as osteosarcomas, neuroblastomas and lung carcinomas, but also in actively proliferating endothelial cells in and around tumor tissues. Adenovirus penton base proteins contain the arginine-glycine-aspartic acid peptide sequence (RGD) motif known to bind itself easily with $\alpha_\beta_\beta_\beta$-integrin. The Adenovirus (Ad) proteins were covalently coupled to AlPcS$_4$ using one or two sulfonamide-hexamido-amide spacer chains (Fig. 5), compounds 8 and 9 [62], using the standard procedure. Ad type 2 structural proteins, the hexon, penton base and fiber antigens were isolated from cell lysate and purified using ion-exchange liquid chromatography and western blot analysis was achieved for checking the protein content. The Ad-AlPcS$_4$ derivatives were tested both in vitro and in vivo. The penton base-AlPcS$_4$ conjugated via two linkers (compound penton-

Pc9 was the most efficient in vitro, as measured in two positive cell lines (A549, Hep2) expressing integrins (light dose yielding 50% growth inhibition (LD$_{50}$) = 18 and 13 J.cm$^{-1}$ respectively versus 25 J.cm$^{-1}$ for murine mammary carcinoma cells (EMT-6) as control cell line) [63]. In vivo, using Balb/c mice bearing EMT-6 tumors in order to target only endothelial cells lining the blood vessels feeding the tumor cells, a mixture of adenosinol soluble proteins-Pc9 required 0.5 $\mu$mol.kg$^{-1}$ to induce the same response with the same light dose, suggesting that the high affinity RGD/receptor complex is able to target Pc9 for PDT [63].

According to this study, it appears that adenoviral proteins can be used to target tumor cells, despite a non-optimal photodynamic activity, and in vivo results were encouraging. Nevertheless, tumor targeting using adenoviral protein vehicles may promote inflammation and anti-protein cellular immunity which could limit their usefulness.

4.4. Monoclonal Antibodies (MAbs)

Photoimmunotherapy (PIT), which combines phototoxicity of the photosensitizers with the selectivity of monoclonal antibodies (MAbs) directed against tumor-associated antigens, has been developed for about 20 years in order to improve the tumor selectivity of the photosensitizers [68], and for the treatment of large surface areas where normal tissue toxicity becomes dose-limiting. Expression of tumor-associated antigens on normal tissues is limited and therefore it was anticipated that these tissues would be spared using MAbs-PS conjugates. In phthalocyanine chemistry, among the hydrophilic photosensitizers suitable to couple MAbs, AlPcS$_4$ is the best adapted compound (only one example of covalent conjugation of ZnPc bearing alcoxy substituents to monoclonal antibodies via an isothiocyanate linker and the use of the adducts as luminescent specific probes has been recently reported [69]).

Several procedures for covalent conjugation of MAbs to Pcs have been described in the literature. Among them, in 1994, Morgan et al. described the coupling of AlPc(5SO$_2$Cl)$_4$ to antibody E7 (MAb E7, IgM subclass) which recognized an antigenic determinant and compared the activities of the covalently conjugated MAb-Pc, using a sulfonamide linker, and of the liposomal MAb-Pc for selective photoimmunotherapy of human bladder carcinoma [70]. They found that covalent conjugation of AlPc(5SO$_2$Cl)$_4$ with tumor-specific antibody E7 widely increased photocytotoxicity compared to free AlPc(5SO$_2$Cl)$_4$. Moreover, photocytotoxicity on human bladder carcinoma cell line 647V expressing the antigenic determinant was dose-dependent for both covalent and liposomal conjugates: at equimolar Pc ratios, 1:1, the MAb-Pc conjugates were 50% growth inhibitory (LD$_{50}$) = 18 and 13 J.cm$^{-1}$ versus 25 J.cm$^{-1}$ for PC$_2$ as control cell line) [63].

In 1999, Carcenac et al. used the same methodology to link AlPcS$_4$ 8 to MAb 35A7 for a selective photoinmunotherapy directed against carcinoembryonic antigen (CAE) [71]. A limited immunoreactivity was observed: the 35A7 MAb-Pc conjugates showed an immunoreactivity close to that of the unconjugated antibody, independently of the number of Pcs anchored on the MAb (5, 12 or 16 mol of Pc per mol of MAb). In vivo, these conjugates were evaluated in nude mice bearing the human colon carcinoma xenograft line T380 and they presented a biodistribution closed to the one obtained with unconjugated
MAb. Even with a loading of 16 mol of photosensitizer per mol of MAb, they obtained up to 32% injected dose/g tumor uptake, as compared to 35% injected dose/g for the unconjugated MAb. For all the conjugates, the tumor-to-normal tissue ratios were as high as those obtained with the unconjugated MAb. More recently, in order to analyze the potential role of internalization of the PS on the photocytotoxicity, they coupled the same Pc to an internalizing antibody, FSP77, directed against ErbB2 and they compared its photocytotoxicity to that of the previous non-internalizing 35A7 MAb-Pc conjugate on an original cell line, SKOv3-CEA-1B9 which expresses both target antigens, i.e. CEA and Her-2, at the same level [72]. The ErbB2 proto-oncogene encodes Her-2 tyrosine kinase receptor, which is frequently amplified and overexpressed in human tumors. By comparison, 35A7 MAb-Pc induced 68% growth inhibition after a 20h incubation time but at a dose as low as 0.04 µg/ml, mean while FSP77 MAb-Pc induced 51% growth inhibition after the same incubation time but at a dose as low as 0.04 µg/ml, which demonstrated clearly the advantage of an internalizing over a non-internalizing MAb-Pc conjugate in terms of photocytotoxicity.

In the same time, Vrouenraets et al. described an original and efficient four-steps synthetic route for the covalent coupling of AlPeS₃ to MAbs U36, E48 and 425 via the tetracycline derivative AlPe(SO₂N₃gly)₄ [73]. Briefly, AlPe(SO₂Cl)₄, prepared by chlorination of AlPe(SO₃H)₄, was converted to the tetracycline derivative AlPe(SO₂Ngly)₄, using a large excess of glycine and benzotriazole (BTA), followed by an esterification of the carboxylic group using a large excess of 2,3,5,6-tetrafluorophenol (TFP) at pH 5.8. Selective hydrolysis and conjugation to MAbs were achieved in a one-pot procedure by dissolving the tetraester in acetonitrile and adding ¹²⁵I-labeled MAb to afford conjugate 10 (Scheme 2). Apparently, due to the MAb concentration used, the three remaining ester groups were hydrolyzed before a 2nd MAb molecule could bind itself to the Pc, which allows the selective formation of 10. Note that they described this new procedure because, according to these authors, standard procedure based on an aminohexanoic linker from Morgan et al. [70], led to unstable conjugates.

All MAbs-Pc drugs presented an increased photocytotoxicity compared to the free Pc. The 425 MAb-Pc with an internalizing antibody showed the highest photocytotoxicity using A431 cell line and a selective tumor targeting in nude mice. In an extended in vitro evaluation, the same authors compared the photocytotoxic activities of analogous mTHPC- and Pc-MAbs conjugates using five head and neck squamous cell carcinoma cell lines as targets and three MAbs (BIWA 4, E48 and 425) [74]. This study demonstrated the high potential of Pc-MAb conjugates in comparison with mTHPC-MAb conjugates for use in PDT. Actually, whereas Pc in free form was not effective, it became highly effective after its coupling to tumor-specific MAbs [74].

5. PHthalocyanines conjugated with steroid hormones

5.1. Cholesterol

The steroid hormones are all derived from cholesterol. They are an interesting class of biomolecules to target PCs to cancer cells. In particular, as previously described, cholesterol is a vital component of eukaryotic cell membranes and can be taken

![Scheme 2](image-url)
up quickly by cancer cells [75]. It thus appears that the covalent coupling of cholesterol to a phthalocyanine molecule could favor its association with LDL and increase its photodynamic efficiency. Furthermore axial substitution of suitable central metals (Si, Ge, Al) by cholesterol, which is a lipophilic and bulky ligand, could decrease Pc self-aggregation (inhibition of the \( \pi-\pi \)-stacking by addition of steric hindrance on the sides of the macrocycle) and increase the crossing of the lipophilic membrane.

Based on this approach, mono and dicholesteryl-substituted MPcs 11 have been prepared (Fig. 6) and incorporated in a monomeric state into unilamellar liposomes [76-81].

Photodynamic activities of the new Chol-Pcs derivatives were investigated on two pigmented melanoma cell lines: M3Dau and SK-MEL-2. M3Dau cells were of interest as they led to melanoma tumors in nude mice, allowing further in-vivo validations and SK-MEL-2 cells were added in order to elucidate whether the photocytotoxicity of the Pcs studied could vary depending on the pigmented melanoma cell line used. The bis(cholesteryloxy) derivative (Chol-O-SiPc, 11d) displayed in vitro the best photokilling efficacy and was seven to nine times more potent than chloro-aluminum Pc (ClAlPc) used as a reference. However, grafting a diphenyl siloxy group onto the cholesteryl moiety (compound Chol-O-SiPh \( \text{O-SiPc} \), 11c) cancelled the gain in efficiency obtained with 11d, which suggests that other parameters such as the nature of the axial ligand play an important role in the photodynamic activity and in the activation of apoptosis [76].

Segalla et al. demonstrated that GePc injected systemically into mice bearing an intramuscularly implanted MS-2 fibrosarcoma was quantitatively transferred to serum lipoproteins and localized in the tumor tissue. The selectivity of tumor targeting by GePc, as it was expressed by the ratio of Pc concentration in the MS-2 fibrosarcoma to the muscle, was similar to that observed for other liposome-delivered Pcs [82]. This could be related to the fact that the presence of two cholesterol moieties brings about only a marginal increase (27% versus 20-24% in the fraction of LDL-bound GePc as compared with other hydrophobic porphyrin or Pc derivatives [83]). On the contrary, the addition of cholesterol to the liposome carrier has been shown to increase up to 33% the amount of Zn(II)Pc associated with LDL [84]. Indeed, it appears that modification of the photosensitizer has a relative minor effect on its distribution among the components of the lipoprotein family; rather, the photosensitizer transfer to lipoproteins is mainly controlled by the properties of the delivery systems. The red-light irradiation of the GePc-loaded fibrosarcoma caused a fast and massive tumor necrosis, involving both malignant cells and blood vessels [80]. The combination of a high photosensitising activity and high affinity for the fibrosarcoma explained the interesting PDT efficacy of GePc.

Another grafting of cholesterol was achieved by Maree et al. who prepared an octasubstituted symmetrical ZnPc linked to the phthalocyanine via a benzoic ester group [85]. They performed the photophysical study and showed that unfortunately it was highly aggregated at low concentrations with a singlet oxygen quantum yield \( \Phi_\Delta = 0.44 \) in CHCl\(_3\) using ZnPc as a reference (\( \Phi_\Delta = 0.55 \) in CHCl\(_3\)). No biological evaluation has been performed on this compound yet.

5.2. Estradiol and Estrone

Always in the aim to improve the uptake of the Pc by receptor-rich endocrine tumors, van Lier et al. have reported the synthesis of phthalocyanine-estradiol conjugates, using an original strategy based on the palladium-catalyzed Sonogashira coupling reaction [86, 87], and their photocytotoxic activities. Thus they prepared a series of lipophilic trit(tert-butyl) Pc-estradiol conjugates (Scheme 3) and compared their biological activities with those of hydrophobic water-soluble trisulfonated conjugates (Scheme 4) [86].
The Pc-estradiol conjugates were prepared with both aliphatic and aromatic alkynyl spacers in order to modulate the overall amphiphilicity of the molecule. Photocytotoxic activity and relative binding affinity for estrogen receptors were measured on murine EMT-6. Lipophilic conjugates 12-14 were photo-inactive up to 1 µM, but they exhibited dark toxicity at 5 µM. The highest receptor binding affinities were observed with the sulfonated analogues coupled via a relatively long spacer while the most hydrophilic trisulfonated Pc 16b and 17a presented the highest photocytotoxicity, especially after 24h incubation (L.D.₉₀ = 2.9 and 3.0 J/cm², respectively), comparable to those reported for the non-conjugated ZnPc₃.

Surprisingly, the nature of the spacer did not seem to influence the biological activity.

Recently, grafting of estrone, the biosynthetic precursor of estradiol, the receptors of which are over-expressed in breast cancer cells, was achieved by Maree et al. who prepared a series of octasubstituted symmetrical GePc and SnPc and axially disubstituted symmetrical SnPc linked to the phthalocyanine via etherification [88]. From the photophysical point of view, aggregation was prevented due to the presence of axial ligands in all complexes. The Φₐ values increased with the increase of the size of the central metal (Si<Ge<Sn), but not much difference in the Φₐ values was observed when estrone was located in axial position or as a substituent on the Pc ring. From the biological point of view, the octaestrone GePc shows the best promise for both i) detection through fluorescence and ii) treatment of tumor cells since this complex has the longest triplet life.

6. PHTHALOCYANINES CONJUGATED WITH OTHER BIOMOLECULES

6.1. Biotine

In order to modulate the hydrophilic/hydrophobic balance and to obtain an optimal amphiphilicity, Photosens® which corresponds to a mixture of aluminium di- (30%), tri- (50%) and tetra- (20%) sulfophthalocyanines bearing hydrophilic sulfonate substituents, was coupled with biotine via a hexanoic linker (Scheme 5) [89]. Biotin, usually called H vitamin or B8 vitamin, is brought by the food and is naturally occurring into human body. The introduction of two biotin substituents onto AlS₃Pc largely increases the lipophilicity of the Pc.

The conjugated photosensitizer 18 was first tested in vitro against monolayers of OAT-75 human breast cancer cells. They observed a LD₅₀ for dibiotinylated Pc 20 times lower than AlS₃Pc, without any dark toxicity. The results of the tests performed in vivo on mice bearing Ehrlich carcinoma provided a much more efficient tumor growth inhibition for dibiotinylated Pc compared with Photosens®. The morphological study of tumor sections had revealed high level of cell death, pronounced vascular damage, and inhibition of tumor cell proliferation activity even at the dose as low as 0.25mg/kg. The application of the dibiotinylated Photosens® at the dose of 0.25 mg/kg sufficiently decreased the area of surviving tumor cells (6 times as compared with Photosens® from 30% down to 5%), increased the extent of necrotic area from 70% up to almost

Scheme 3. Preparation of estradiol-containing Zinc(II) tri(tert-butyl)-phthalocyanines.
total (95%), and the level of apoptosis exceeded 50%. All those improvements could be tentatively explained by an easier penetration of the drug within the cell membrane and its favored accumulation inside at high concentrations. Moreover, it is interesting to note that the proliferative activity of the tumor cells decreased from the moderate down to the weak, the desmoplastic reaction extent changed from the moderately expressed (detection of capsule’s fragments around tumor, so called pseudo-capsule) down to the weakly expressed (proliferation of myelofibroblasts on the tumor border).

6.2. Oligodeoxyribonucleotide and Nucleobases

On the contrary of the large number of nucleobase-porphyrins, which are of interest for antiviral and anticancer therapies [90, 91], there are only five examples of phthalocyanine-nucleobase dyads described in the literature. Koval et al. have first reported in 2001 the synthesis of oligonucleotide-phthalocyanines conjugates for specific DNA modifications in vitro and in vivo, which is of interest for the development of sequence specific gene targeting reagents [92]. Later on, Hammer et al. prepared asymmetrical water-soluble Pcs 19-20 conjugated with an oligonucleotide via an isothiocyanate function (Fig. 7). Those dyes present favorable photophysical properties and excellent water solubility which make them potentially excellent fluorescent tags for genetic assays [93].

The same year, Li et al. prepared the first adenine-phthalocyanines adducts 21 by standard O-alkylation of the tetrahydroxy-phthalocyanine (Scheme 6) [94].
Due to the presence of a phthalocyanine core and adenine substituents, these compounds present strong intermolecular interactions ($\pi-\pi$ stacking, intermolecular H-bonding and intermolecular axial coordination of an adenine moiety to an adjacent Zn(II) center forming a pseudo-dimer), resulting in a poor solubility in common organic solvents and unusual spectral features (observation of fluorescence emission despite strong aggregation). No biological evaluation was performed.

Very recently, in order to modulate the aggregation/de-aggregation properties of Pcs, Sessler et al. also prepared a phthalocyanine-linked cytidine derivative 22 (Fig. 8) and studied its supramolecular behaviour using UV-Vis and fluorescence spectroscopy [95].

No PDT evaluation has been performed with those nucleobase-Pcs adducts, whose aggregation severely limits their use in biological medium. Note that to overcome this problem of strong aggregation, Reddy et al. have prepared very recently similar Pcs-nucleobase conjugates but using zinc phthalocyanines with 12 trifluoroethoxy substituents instead of the 3 $t$-Bu substituent; this largely increases their solubility and reduces the aggregation as shown by UV-Vis spectroscopy. Evaluation of the PDT behaviour of those compounds is under investigation [96].

CONCLUSION

This review presents, to the best of our knowledge, all biological molecules, or molecules of biological interest, which have been covalently coupled to phthalocyanines. An important determinant of successful PDT targeting is the localization of the photosensitizer in neoplastic tissue. Although most of the photosensitizers in their currently used formulations provide adequate selectivity for the limited indications, the reach and the ease of use would be greatly enhanced if significantly high selectivity accumulation in tumor tissues could be achieved. The rationale for the use of molecular delivery systems for photosensitizers like phthalocyanines is similar to that for the delivery of chemotherapeutics and toxins. There are however two main differences in the requirement in the photo-and non-

photon-based approaches. First, in conventional therapy the drug has to be freed to elicit the appropriate biologic response. Certainly, this is not a prerequisite when carrier molecules are used for delivery of photosensitizers. Second, in PDT, the requirements for specificity of the delivery molecule such as phthalocyanines are less constraining. This is a consequence of the inherent selectivity of the light delivery. Nevertheless, the problems associated with the use of large molecules, such as complicated syntheses, transport barriers, and potential systemic toxicity, are similar for photo-conjugates like conjugated phthalocyanines and for other conjugates. The diversity of cellular and tumor tissue characteristics will probably lead to the discovery of appropriate photosensitizer targeting mechanisms. Research is ongoing to find the infallible “magic bullet”. However, a less general approach could probably be more realistic as each cancer phenotype must be targeted on an individual expression.
Phthalocyanines are extremely powerful photosensitizers and pre-clinical work clearly shows their immense promise in PDT. However, only few of these photosensitizers were tested in vivo for PDT treatment, which is in part due to the high complexity of phthalocyanine chemistry in terms of synthetic yields and workup compared to other porphyrinic macrocycle chemistry (general routes to water-soluble Pcs containing a labeling function have not been reported to date because of the need for preparing asymmetrical Pcs, which can be very difficult to purify, due to statistical formation of isomers during macrocycle assembly), but also to their important tendency to self-aggregate in biological medium. Nevertheless, a lot of new synthetic pathways of Pcs are currently under investigation, not only to circumvent the purification problems and to increase their absorption in the near-infrared region, but also to get efficient accumulation of the photosensitizer at suscetible subcellular locations. Therefore, photosensitizer conjugates that accumulate in cells within endosomes and lysosomes may be less effective than the corresponding non-conjugated photosensitizer despite increased intracellular uptake.

References
