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ARTICLE TYPE

Antibodies armed with photosensitizers: from chemical synthesis to photobiological applications

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Targeting photosensitizers to cancer cells by conjugating them with specific antibodies, able to recognize and bind to tumor-associated antigens, is today one of the most attractive strategies in photodynamic therapy (PDT). This comprehensive review updates chemical routes available for the preparation of photo-immunoconjugates (PICs), which keep the dual chemical and biological functionalities: photo-properties of the photosensitizer and immunoreactivity of the antibody. Moreover, photobiological results obtained with such photo-immunoconjugates using *in vitro* and *in vivo* cancer models are also discussed.

1. Introduction

Molecular targeted photodynamic therapy (PDT) relies on the conjugation of a non-toxic photosensitizer (PS) with a biomolecule able to target cancer cells.[1-6] Monoclonal antibodies (mAb) seem to be ideal carriers for PSs, due to their ability to recognize a small portion of several "non-self" molecular configurations which are conventionally termed antigens. As mAbs are able to target antigens expressed on malignant cells, they can be used as vehicles to deliver a PS selectively to the cancer cell.[2,3] In targeted PDT, the selective uptake of the PSs by cancer cells *via* proteins overexpressed at the cell surface, followed by light irradiation can induce photo–physical and –chemical reactions resulting in reactive oxygen species (ROS) production, and subsequent reaction with surrounding biomolecules.[7] PDT has been shown to destroy the tumors by multifactorial mechanisms (cellular, vascular and immunologic) which share a common feature: they are mediated by ROS generation during PDT (Figure 1).[8,9]

Targeting drug delivery through molecular recognition of moieties by carrier ligands is widely accepted for improving the efficiency of therapeutic regimen. The coupling of an antibody with a PS (named photo-immunoconjugate, PIC) was first proposed in the 80s by Mew *et al.*[10] and the better photodynamic efficiency of PICs when compared with the free PSs was demonstrated. Findings in chemically modified or new synthetic PS-drugs, as well as in the field of antibody engineering have contributed to the development of more efficient PICs.[1-3] From a clinical point of view, photoimmunotherapy is a promising approach in cases where the desired selectivity utilizing controlled precise application of light to diseased tissue might not be possible (*e.g.* tumor nodules spreading over a large surface such as the peritoneal cavity, mouth or bladder). Despite of numerous pre-clinical studies to date, there are few studies describing clinical studies with PICs[11-13] and there are no ongoing clinical studies using PICs. Ideally, a PIC should retain the antigenic specificity of the antibody as well as the intrinsic photophysical properties (*i.e.* ability to generate ROS) of the PS. Therefore, the synthetic methodology used in the coupling should not influence the properties of mAb and PS. After the coupling reaction, the efficacy of the new PIC should be validated using specific *in vitro* and *in vivo* models.

This review highlights the most significant aspects on the preparation of PICs for PDT. The chemical strategies (direct or indirect) for the synthesis of PICs are critically examined. The properties of the antibodies and the photo–chemical and –physical properties of the PSs are discussed. Furthermore, this review will summarize the recent progress with respect to the: intracellular accumulation, cellular localization, and cytotoxicity after photoimmunotherapy (PIT), as well as the pharmacokinetic behaviour of PICs. Practical considerations for the development and/or optimizations of efficient PICs will be suggested.

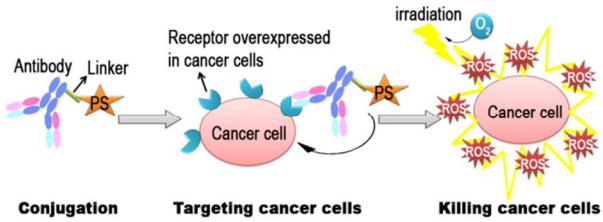


Fig. 1 Photodynamic action of photosensitizers conjugated with antibodies.

2. Photodynamic therapy and the advances in photosensitizer's chemistry

In clinical cancer settings, the PS is typically administered intravenously or topically, followed by light illumination of the anatomical site being treated.[4,14] First, PSs are preferentially taken up by cancer cells. After their accumulation, the tumor is light irradiated. In the presence of molecular oxygen, the combination of PS and light at a specific wavelength can result in the production of singlet oxygen ($^1O_2$) which has been shown to be the main type of ROS responsible for tumor destruction.[1,9,15] Nowadays, the discovery of new PSs with outstanding photo-physical and -biological properties is a lively research field.[4,14,16,17] PSs are different in terms of chemical and biological characteristics and their classification can be based on their generation time: first, second and third generation PSs (Figure 2).[4,18]

The first generation PSs are based on hematoporphyrin and its derivatives. The second generation PSs include benzoporphyrin derivates, chlorins, phthalocyanines, texaphyrins and natural compounds such as hypericin. When compared with first generation PSs, second generation PSs show an absorption spectrum extended to the red and near-infrared regions of the electromagnetic spectrum (600-800 nm), allowing the treatment of deep tumors due to deeper tissue penetration by red light (Figure 2). Furthermore, with second generation PSs, the $^1O_2$ production is markedly improved at these wavelengths compared to the first generation PSs. Knowing that first and second generation PSs are non-selective for cancer cells and can also cause toxicity in healthy cells, third generation PSs were developed. These PSs are first or second generation PSs conjugated to/or introduced into biochemical carriers that allow biological specificity by delivering/targeting such PSs to the cancer cells.[1,19,20]

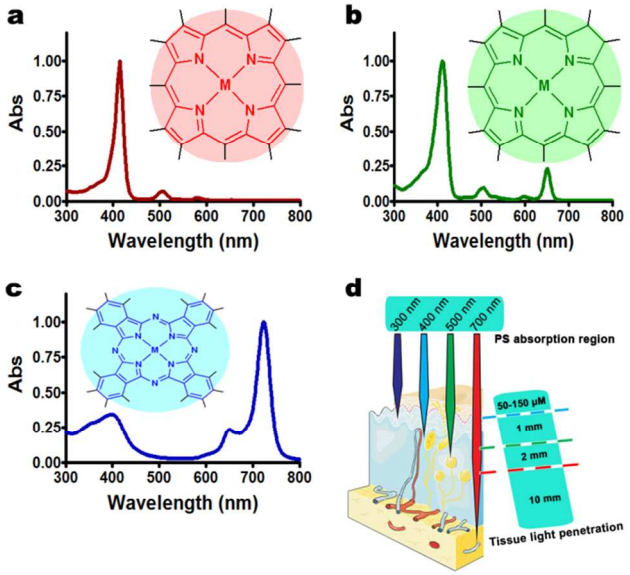


Fig. 2 Porphyrin (**a**), Chlorin (**b**) and phthalocyanine (**c**) structures and their respective absorption spectra.(**d**) Correlation between the absorption of light by the photosensitizer molecule and the penetration of the light through the tissue. Adapted from Agostinis *et al.*[5]

Many hypotheses have been proposed for the development of third generation PSs. Although liposomes and nanoparticles can improve tumor accumulation, the biomolecules with specificity for structural features overexpressed or enhanced in tumor-associated tissue are being conjugated to PSs showing most promising results.[1] Published studies involving photoactive bioconjugates refer mostly to PSs conjugated to mAbs directed against tumor antigens,[21,22] sugars,[23-27] oligonucleotides to enhance photogenotoxicity,[28] hormones, metabolites, cellular signaling species to selectively target an overexpressed enzyme or receptor,[29] and also to peptides,[30] and amino acids.[31]

3. Molecular properties of antibodies

In humans, there are five major immunoglobulin classes or types (IgG, IgM, IgA, IgD and IgE). Amongst the five antibody classes, the gamma globulins (so-called IgG class) are the most abundant ones. IgGs are also smaller and more stable during isolation and purification than the other immunoglobulin classes. Therefore, IgG is the most used immunoglobulin class in the development of antibody-drug conjugates.[32,33] The structure of an IgG immunoglobulin molecule consists of two identical γ heavy chains and two identical light chains (termed κ or λ), which are linked together by inter-chain disulfide bonds.[34] The *N*-terminal domains of each light and heavy chain have variable amino acid sequences, and are thereafter referred to as the variable regions V_L and V_H, respectively (Figure 3). On the other hand, the *C*-terminal domains of heavy and light chains have constant amino acid sequences, and are referred to as the constant regions C_L and C_H, respectively. The interaction between the variable proportions of the V_L and the variable portion of the V_H leads to the formation of two combining sites for antigen binding.

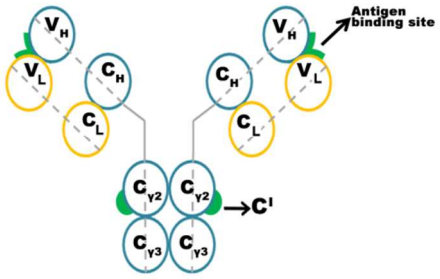


Fig. 3 The domain structure of an IgG immunoglobulin light and heavy chains. The two heavy chains are linked by disulfide bonds. The interaction between the V_H and the V_k lead to the formation of the combining site for antigen binding. The binding site for the complement (C^1) is represented on the C_γ^2 domain. The region of the C_H where no domains are present is termed "hinge" region.

Polyclonal antibodies, monoclonal antibodies (mAbs) and recombinant antibodies/antibody fragments are three types of immunoreagents. Polyclonal antibodies are produced from different B-lymphocyte cell lines and correspond to a heterogeneous mixture of antibodies recognizing different epitopes of the same antigen(s). A mAb is produced by a single B-lymphocyte clone and it is specific for only one epitope on an antigen. Considering the properties of mAbs, they have been selected in their production for their ability to serve as specific binders of the target antigen. Whole IgGs should allow for higher degree of labelling (DOL, *i.e.* number of PS molecules covalently linked per antibody) (Figure 4), since they have more lysine residues available for bioconjugation in comparison with mAb fragments. In fact, previous studies associated higher DOLs with a better PDT ef-

fect. Nowadays, several reports have suggested that high DOLs may disturb the immunoreactivity of the mAb after coupling to PSs. Whole IgGs have a long serum half-life when compared with antibody fragments, which can result in higher non-specific uptake in non-targeted tissues. Moreover whole IgGs bear a crystallisable fragment (Fc) region, which interacts with the complement system and effector cells of the immune system, resulting in immunogenic response induction. Additionally, solid tumors have poor vascularisation what limits diffusion of the mAb through the tumor.

The limitations of mAbs have spurred the production of smaller mAb fragments, which are characterized by faster blood clearance. While mAb fragments have advantages over whole antibodies, their production is time consuming and laborious. It involves techniques of genetic engineering and molecular biology (molecular cloning, protein expression and purification) or digestion with papain or pepsin. Most commonly used in the production of PICs are single-chain variable fragments (scFv, Mw = 25 kDa) and fragment antigen-binding (Fab) regions: F(ab´)$_2$, Mw = 100 kDa and Fab´, Mw = 50 kDa.[33] Another antibody fragment which has been recently used in PICs production is the small immune protein (SIP) format,[33,35,36] which in terms of blood clearance is intermediate between antibody fragments and whole antibodies (Figure 4), resulting in reduced accumulation of PICs in vital organs. Latest reports present PSs conjugation to nanobodies. [73] Nanobodies are variable domains of the heavy chain of antibodies which were firstly discovered in camelid species. They have a unique structure that is devoid of the light chain, having a region that is functional equivalent to the Fab fragment of the conventional antibodies at their N-terminal region.[37] Due to their low molecular weight – 15kDa - and dimensions –4×2.5 nm, they are considered to be the smallest antigen recognizing fragments.

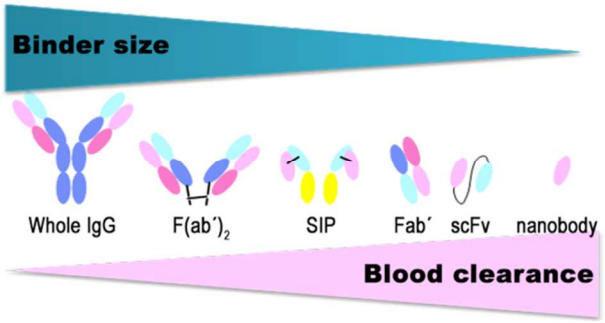


Fig. 4 Antibodies and recombinant antibodies/antibody fragments used in the development of photo-immunoconjugates.

4. Technical aspects of the synthesis of photo-immunoconjugates

During the development of PICs there are several combinations of PSs and mAbs that can be tested (Figure 5). In order to overcome the limited success of PIT (especially when applied *in vivo*) it is important to choose the appropriate type of mAb, synthetic route and type of PS modification. The PS should exhibit outstanding photophysical properties, such as photostability, high ability to generate $^1O_2$ and absorption bands in the red and near-infrared regions of the electromagnetic spectrum (Figure 2). The PS also needs to have a reactive chemical group/linking group for direct or indirect coupling to the amine,[10,35,36,38-52] sulfhydryl[33,53-59] or aldehyde[60-63] groups present or created on the antibody. The mAb should have specific affinity to highly expressed epitopes in the tumors, and low immunoreactivity with the normal tissues.

The bioconjugation strategy employed should provide stable PICs able to be reproduced with the same consistency every time. The presence of light and oxygen during PIC synthesis and purification can result in the generation of ROS, which will affect the integrity of the conjugate. Thus, it is essential to perform all the bioconjugation steps in darkness and with all the solvents saturated with nitrogen.

The coupling reactions between PSs and mAbs can be easily followed by observing the absorbance of the PS (400–800 nm) and the absorbance of mAb protein (280 nm). The DOL of the resulting PICs is determined after its purification by spectroscopy using the molar extinction coefficient of the PS and the mAb concentration determined by protein assay kits based on Bradford or Lowry methods. This DOL PS/mAb can be also determined by mass spectrometry, especially MALDI-TOF/MS.

High-performance liquid chromatography (HPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are the common techniques used for the evaluation of PIC integrity. These methodologies should provide information about the presence of undesirable non-covalently attached PS. After purification and assessment of PIC integrity the next steps consist on the evaluation of its specificity to bind and target the respective antigen and its photophysical efficiency to generate phototoxic reactions. Enzyme-linked immunosorbent assays (ELISA) or immunofluorescence assays can be performed for the validation of the specificity of PIC binding. In order to confirm its binding specificity, the experiments are performed either by pre-incubating the cells with an excess of respective unconjugated mAb or by using antigen negative cells. The immunoreactivity of the PIC can also be determined with binding assays of antigen immobilized on sepharose matrix.[64] The photophysical properties of the conjugated PS, such as solubility, generation of $^1O_2$ and photo-stability can be determined by spectroscopic methods.

In vitro and *in vivo* evaluation of PICs is of paramount importance to validate their photodynamic efficacy. *In vitro* assays are performed by exposing cancer cells overexpressing the targeted antigen for different irradiation times and PIC concentrations. The phototoxicity is evaluated using cell viability assays and comparing the results with valid controls (*e.g.* illumination with no PIC, PDT in cells incubated with free PS, free mAb or non-specific PIC). *In vivo* studies have been performed using specific animal models and enclose biodistribution and phototoxicity studies (measurements of concentration of PIC accumulating in specific organs, volumes of treated and control tumors and plotting survival curves of treated animals). Better understanding of cell death pathways induced after *in vitro* and *in vivo* photo-immunotherapy allows for further improvement of PIC efficiency on molecular level.

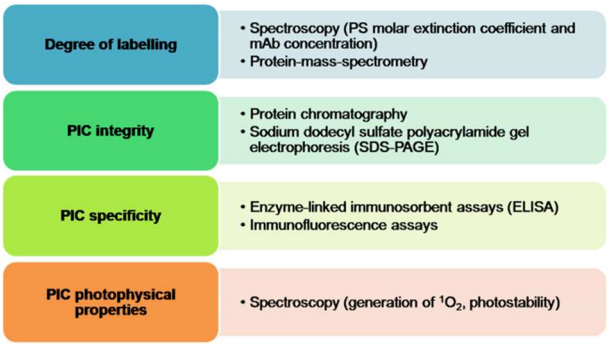


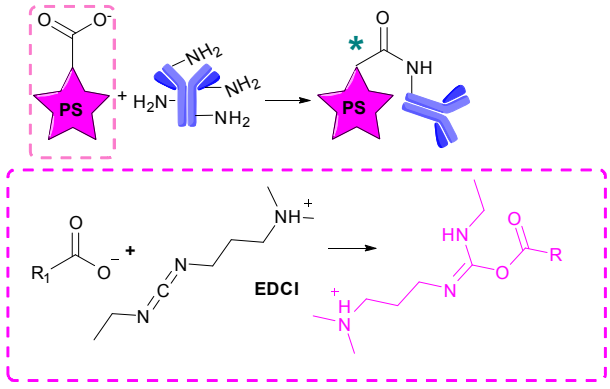
Fig. 5 Technical aspects of the coupling of photosensitizers with antibodies.

5. Synthetic routes in the development of photoimmunoconjugates for cancer treatment using whole antibodies or antibody fragments

The bioconjugation strategy can be performed by direct conjugation using carbodiimide coupling,[10,51,52,65-68] reductive amination,[69] activated esters,[22,35,36,42,46-50,64,70-74] isothiocyanate,[43,45,75,76] maleimide[33], acryloyl functionalities[77]or by classical click-type reactions (copper-catalysed azide−alkyne cycloaddition).[78] The indirect coupling implies the pre-conjugation of PSs with carriers or scaffolds such as dextran,[79-81] polylysine,[56,58,59] *N*-(2-hydroxypropyl)methacrylamide,[82] polyvinyl alcohol[40,41] or polyglutamic acid.[60,62] The indirect conjugation can also be performed by pre-coupling of mAb onto polyethylene glycol linker.[83] The coupling of photosensitizers with lysine groups (which at can be located at the antigen binding site) can result in loss of antibody immunoreactivity. This problem can be avoided by coupling photosensitizers with groups located at the stalk or hinge region of the antibody (*e.g* reduced disulfide bonds or oxidized carbohydrate groups). Amongst direct bioconjugation strategies, reductive amination and acryloyl functionalities have not allowed the synthesis of promising PICs to be applied as photodynamic agents in cancer treatment. Therefore, these two bioconjugation strategies will not be highlighted in this report. The most common bioconjugation strategies used in the development of PICs for cancer treatment are discussed in the following sections.

5.1. Conjugations *via* carbodiimide coupling

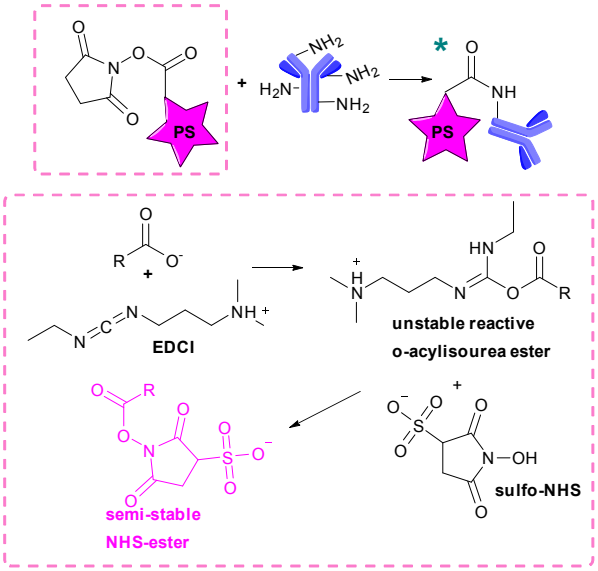
The carbodiimide coupling relies on the use of a carbodiimide reagent,1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloric acid (EDCI), that reacts with carboxyl groups of the PS (Scheme 1) to produce the key intermediate (*O*-acylisourea).[10,51,52,65-68] The antibody is then added to the activated PS and bioconjugation occurs in a buffered solution involving the formation of amide bonds between the amine groups on the antibody and the carboxy groups of the PS. Mew *et al.* were the first ones reporting the conjugation of a mAb with hematoporphyrin *via* carbodiimide coupling.[10] One of the major drawbacks associated with this synthesis is the presence of non-covalently bound PS in the PIC even after purification.[10]



Scheme 1 Carbodiimide coupling used in the coupling of PSs with antibodies (*means representation of a possible conjugate).

5.2. Conjugations *via* activated *N*-hydroxysuccinimide (NHS) ester or sulfo-NHS

The problems associated with the carbodiimide strategy led to the development of PSs containing activated esters. With this method, the carboxy groups of the PS are transformed into *N*-hydroxysuccinimide esters after treatment with *N*-hydroxysuccinimide (NHS) in the presence of *N,N′*-dicyclohexylcarbodiimide (DCC) analogues. The PS can then be isolated and purified before conjugation with amino functionalities on the mAb forming an amide bond (Scheme 2). The PS containing NHS esters can be also formed *in situ* from a carboxylate by coupling the aforementioned carbodiimide reaction with the addition of NHS ester (Scheme 2).



Scheme 2 Coupling of photosensitizers with antibodies *via N*-hydroxysuccinimide (NHS) ester or sulfo-NHS (*means representation of a possible conjugate).

Bhatti *et al.* reported the coupling of verteporfin succinimidyl ester (verteporfin-NHS) with scFv fragments.[49] The conjugation of verteporfin-NHS with scFv fragments containing less lysine residues than scFv resulted in PICs with low photophysical characteristics, demonstrating that both the number and position of lysines have implications on the design of new PICs.[49] Recently, the NHS ester of the commercially available silicon phthalocya-

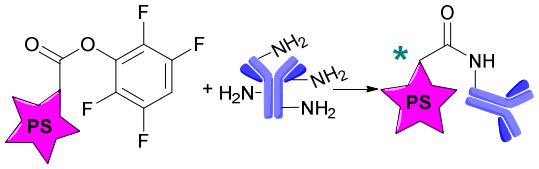
nine derivative (described as the near-infrared fluorescent PS, IRDye700DX) has been conjugated with whole antibodies (trastuzumab,[21] panitumumab[21] and anti-human carcinoembryonic antigen[74]) or with nanobodies[72] targeting epidermal growth factor receptors. After conjugation, PICs retained the immunoreactivity of the antibody and the DOL was lower with nanobodies than with whole antibodies.[21,72]

Carcenac *et al.*[50,64] have used the carbodiimide method described by Brasseur *et al.*[70] to conjugate the tetrasulfonated aluminium phthalocyanine ($AlPcS_4$) with whole mAbs overexpressed in foetal colon and colon adenocarcinomas, breast and ovarian cancers. The first step in the synthetic procedure was the conversion of the $AlPcS_4$ sulfonic acid groups into sulfonyl chloride functionalities after treatment with thionyl chloride.[50,64,70] The AlPc-tetrasulfonyl chloride allowed the reaction with 6-aminohexanoic acid and sodium carbonate to yield the monosulfonamide product bearing a single carboxyl group. After purification, the carboxylic acid moiety in the phthalocyanine was activated as carbodiimide by treatment with EDCI and sulfo-NHS. The activated PS was added dropwise to a buffered solution of mAb.[50,64,70] The degree of labeling for these PICs was 5, 12 and 16 by using initial molar ratios (moles of activated PS per mole of mAb) of 20, 40 and 80, respectively. The PICs also contained a certain percentage of aggregates, which increased from 1, 1.12 to 1.18 for conjugates with a degree of labeling of 5, 12, and 16, respectively. The amount of such aggregates was reduced after removal of excess carbodiimide (prior bioconjugation).

Fabbrini *et al.*[36] have conjugated SIP and scFv fragments with bis(triethanolamine)-Sn(IV)chlorin e6-NHS (**SnChl$_{e6}$**-NHS) formed *in situ* by coupling the carbodiimide reaction with the addition of sulfo-NHS to **SnChl$_{e6}$**. Palumbo *et al.*[35] also synthesized SIP PICs in an attempt to obtain a selective destruction of tumor neovasculature. 5-(4-Carboxyphenyl)-10,15,20-tri-(4-pyridyl)porphyrin was used as starting material to introduce an activated ester group. First, the carboxylic acid groups were converted into acyl chloride functionalities after reaction with thionyl chloride. Next, porphyrin reacted with NHS to introduce an activated ester group that allows for bioconjugation. On the final step, tricationic porphyrin was obtained after reaction with methyl iodide. Herein, the conversion of the counter ion from iodide to chloride increased water solubily as demonstrated previously by Sutton *et al.*[42]

5.2.1. Conjugations using esterification of carboxylic acid groups on PS to tetrafluorophenyl esters

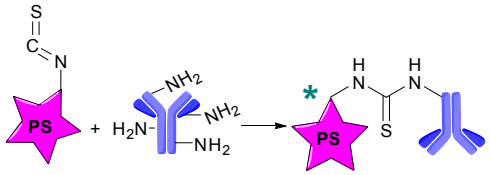
Vrouenraets *et al.* have developed bioconjugation strategies to couple the poorly water soluble *meta*-tetrahydroxyphenylchlorin (*m*THPC) with mAbs.[46] The commercially available *m*THPC (Foscan, Temoporfin) was radiolabeled and then tetracarboxymethylated using iodoacetic acid. Methylation increased *m*THPC hydrophilicity and resulted in the formation of functional groups suitable for conversion into activated esters. The four carboxylic acid groups were esterified using 2,3,5,6-tetrafluorophenol and EDCI (Scheme 3). The conjugation of the ^{131}I-labeled PS with the respective ^{125}I-labeled mAb was performed after partial hydrolysis of the activated ester. In another experiment, it was observed that successive additions of PS resulted in an increase of the ^{125}I:^{131}I molar ratio, however formation of mAb aggregates was observed. The synthetic procedures developed by Vrouenraets *et al.* also proved that bioconjugation strategies developed under darkness and using solvents saturated with nitrogen contribute to the maintenance of mAb integrity. Vrouenraets *et al.* developed further studies using aluminum(III) phthalocyaninetetrasulfonate [$AlPc(SO_3H)_4$] that is a hydrophilic PS with absorption bands around 675nm appropriate for deeply localized tumors treatment,[73] and 5-[4-[5-(carboxyl)-1-butoxy]phenyl]-10,15,20-tris(*N*-methylpyridinium-4-yl)porphyrin iodide(TrisMPyP-ϕCO_2H),[47] a porphyrin derivative more hydrophilic than *m*THPC due to its three methyl-pyridinium moieties.



Scheme 3 Coupling of tetrafluorophenyl PS esters with antibodies (*means representation of a possible conjugate).

5.3. Conjugations using isothiocyanate (NCS) functional groups

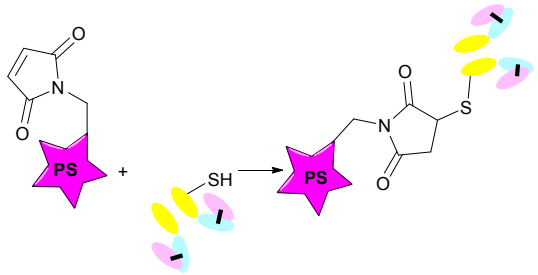
The use of isothiocyanate (NCS) functional groups has advantages over coupling *via* carbodiimide or NHS functionalities, since it allows bioconjugation under soft conditions and without formation of intermediates or by-products. The NCS functionalities of the PSs react with amino groups on the mAb forming an isothiourea bond (Scheme 4). Malatesti *et al.*[43] have reported an efficient method for conjugation of cationic 5,15-diphenylporphyrins containing a single isothiocyanate group with mAbs. The synthesis of the porphyrins was based on the method described by Sutton *et al.*,[42] who used PSs containing a single amine-reactive isothiocyanate group to conjugate with bovine serum albumin. The cationic PSs were derived from porphyrins containing a protected amino group, and one pyridyl group, or a dimethylamino group. The isothiocyanate functionality obtained after treatment with 1,1´-thiocarbonyldi-2,2´-pyridone was then conjugated with mAbs. The degree of labeling was dependent on the porphyrin used and it was higher for porphyrins containing a dimethylamino group. This strategy has also been used by Smith *et al.*[45] and Hudson *et al.*[38] to conjugate mono-cationic and tricationic porphyrins bearing an isothiocyanate group with mAbs. The capability of porphyrins containing only one isothiocyanato group to be conjugated with mAbs, combined with their exciting *in vitro* results,[38] prompted Duan *et al.*[75] to conjugate a phthalocyanine bearing a single isothiocyanato group with mAbs. The degree of labelling obtained after purification of phthalocyanine-immunoconjugates[75] was higher than those obtained for porphyrin-immunoconjugates.[43,45] Recently, isothiocyanate conjugation route has been applied with a porphycene.[76] Porphycenes have advantages when compared with porphyrins, since they have high absorption in the red spectral region.[76]



Scheme 4 Coupling of PSs with antibodies using isothiocyanate (NCS) functional groups (*means representation of a possible conjugate).

5.4. Conjugations using maleimide functional groups

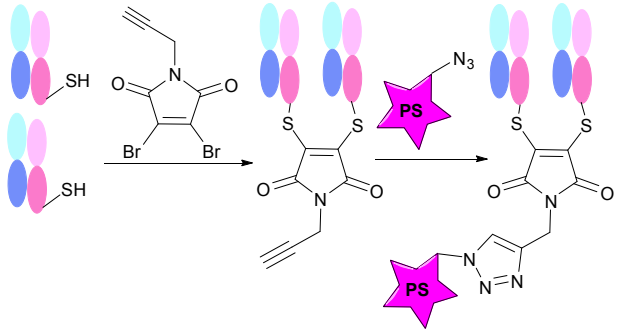
In this methodology, maleimide substituted PSs are conjugated with mAbs containing a sulfhydryl reactive group, forming a thioether bond (Scheme 5). Alonso *et al.*[33] developed a specific coupling of SIP to PSs by conjugating porphyrins containing a maleimide group with *C*-terminal cysteine residues present at the end of the SIP. The synthesis of the maleimide porphyrin derivatives was accomplished by two different synthetic routes. In the first methodology, the porphyrin with an amino group was reacted with maleic anhydride to afford the maleic acid intermediate. The required maleimide-porphyrin was then achieved after cyclodehydration of the intermediate. In the second methodology, the carboxylic acid groups in porphyrins were converted into the corresponding acid chloride *via* acyl chloride. Then, NHS was added to generate porphyrins containing an activated NHS ester group. Porphyrins bearing an activated NHS ester group were reacted with 1,6-diaminohexane or *O,O'*-di-(2-aminoethyl)-hexaethylene glycol which had one amino group Boc (*tert*-butyloxycarbonyl) protected. The maleimide porphyrins were obtained after acid hydrolysis (to remove the Boc protecting group) and further reaction with the hetero-bifunctional cross-linker succinimidyl-4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate in dry *N,N*-dimethylformamide in the presence of *N,N*-diisopropylethylamine. The final step in both methodologies involved the quaternization of the pyridyl groups in maleimide porphyrins with methyl iodide. The optimal reduction conditions to reduce the cysteine residues of the *C*-terminal region of the SIP fragment (without disturbing the intra-domain disulfide bridges) were achieved using tris(2-carboxyethyl)phosphine. The PICs corresponding to porphyrin linked directly to SIP(L19), porphyrin with a small hydrocarbon spacer and porphyrin with a long hydrocarbon spacer were obtained with DOLs of 0.76, 0.90 and 1.75, respectively.



Scheme 5 Coulpling of photosensitizers with SIP antibody fragments using maleimide functional groups.

5.5. Conjugations *via* click chemistry

The efficiency and specificity of click chemistry, as well as the high yields obtained with this conjugation strategy have motivated its use in the development of PICs. The most well known example of a click reaction is the copper-catalyzed azide-alkyne cylcoaddition, which yields a 1,4-disubstituted five-membered 1,2,3-triazole ring (Scheme 6). Recently, Bryden *et al.* reported a new and simple method to couple azide-functionalized porphyrins with trastuzumab Fab fragments, which allows the synthesis of homogeneous products (Scheme 6).[78] A Fab fragment was generated after successive digestions of trastuzumab with pepsin and papain. After reduction of the interchain disulfide bridge of the Fab fragment, it was treated with *N*-propargyl-3,4-dibromomaleimide which allowed their conjugation with porphyrin. The PIC was obtained by treating the functionalized Fab fragments with azide-bearing water-soluble porphyrins.



Scheme 6 Coupling of photosensitizers with Fab antibody fragments using a click-type reaction.

5.6. Conjugations using polymeric linkers

The use of the aforementioned direct bioconjugation strategies results occasionally in the formation of bioconjugates with PS molecules in close proximity to each other (decreasing the ability of them to generate ROS) and in undesirable conjugation of PSs in the mAb recognition site (decreasing immunoreactivity). The drawbacks associated with direct bioconjugation strategies have spurred the development of indirect strategies using linkers (such as dextran,[79-81] polylysine,[56,58,59] *N*-(2-hydroxypropyl)methacrylamide,[82] polyvinyl alcohol[40,41] or polyglutamic acid[60,62]) to preload the mAb or the PS molecule before bioconjugation (Figure 6). These coupling methodologies have allowed the synthesis of PICs with improved solubility, stability and preserved immunoreactivity.

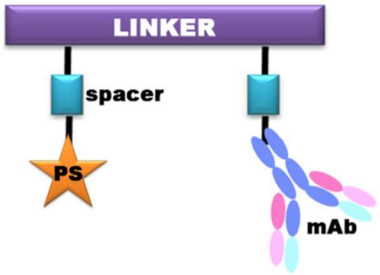
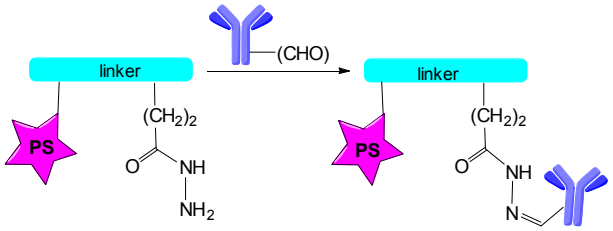


Fig. 6 Coupling of PSs with antibodies using polymeric linkers.

Although improvements have been achieved with polymeric linkers, the coupling of benzoporphyrin derivative (BPD) verteporfin with polyethylene glycol resulted in lower uptake and phototoxicity when compared with free BPD verteporfin.[44] The

PIC demonstrated accumulation in lysosomes and endosomes[84] while free hydrophobic BPD verteporfin was accumulated in lipophilic compartments of the cells like mitochondria and perinuclear region. The same PIC was also evaluated by Abu-Yousif *et al.* who demonstrated that it undergoes vesicle mediated transport into lysosomes, which is characteristic for unconjugated mAb.[84] The use of the polyglutamic acid linker functionalized with hydrazine has been used to couple **Chl_{e6}**-monoethylenediamine monoamide derivative (**Chl_{e6}-MA**) with the carbohydrate moieties (at the hinge region) of several antibodies (Scheme 7).[60,62] Herein, the mAb previously oxidized by reaction in sodium periodate is added to the functionalized **Chl_{e6}-MA**-polyglutamic acid bearing amino groups. The bioconjugates were obtained with a degree of labelling of 20 **Chl_{e6}-MA** per linker and one mAb per linker. Unfortunately, the bioconjugates demonstrated a certain percentage of non-covalently bound **Chl_{e6}-MA**, as well as some evidence of cross-linking.



Scheme 7 Coupling of photosensitizers with the carbohydrate moieties (at the hinge region) of antibodies *via* polyglutamic acid linker functionalized with hydrazine.

6. Photo-immunoconjugates as photodynamic agents using *in vitro* and *in vivo* models

The next subsections aim to describe *in vitro* and *in vivo* photodynamic activity of many PICs. Since the first scientific reports including biological evaluation of PIC were published in the early 80s,[10] biological studies with PICs have increased dramatically. However, currently, there are no ongoing clinical trials of PICs for cancer treatment. Nevertheless, further progress in PIC application is expected due to the latest clinically approved antibody-drug conjugates and reports evidencing efficacy of PIT.[22,35,71,72]

6.1. *In vitro* studies with photo-immunoconjugates

6.1.1. Photo-immunoconjugates immunoreactivity

The commonly used method for PS conjugation with proteins involves random coupling of amino groups of lysine residues and sulfhydryl groups of cysteine residues present in mAb with reactive groups of the PSs. While whole mAbs contain more lysine residues than mAb fragments, the possible loss of mAb immunoreactivity is higher with mAb fragments than with whole mAbs. Genetic modification of mAb by removing lysine residues in the antibody binding site has been suggested as one of the strategies to overcome the problem of immunoreactivity loss after coupling with PS.[85] Nevertheless, it is necessary to certify whether the removal of lysine residues in the antigen binding site has no influence on binding ability of the antibody with the epitope of the antigen. Another attractive bioconjugation strategy is coupling of PSs to cysteine residues which unlike lysines are remote from the antigen binding site and due to fact that the number of cysteines in amino acid sequences of mAbs is lower than those for lysines, providing more predictable sites of conjugation.[33] However in both cases PSs can be attached in the antigen binding site of mAb, resulting in decrease or loss of immunoreactivity after conjugation. For further biological studies and pharmacological applications it is essential to prove that after coupling, the mAb maintains its ability to recognize and to bind the epitope of the antigen.

Immunoreactivity tests are based on the comparison of biding of the PIC with the unconjugated mAb (which serves as control) and an irrelevant antibody (which does not recognize the antigen of interest).There are several reports indicating that high DOLs are associated with a decrease in PICs' immunoreactivity.[36,46] Duska *et al.* have demonstrated that the immunoreactivity of anionic modified PICs is not changed while cationic PICs showed an increase in immunoreactivity.[54] These results are supported by Del Governatore *et al.*,[57] who investigated PIC bearing polylysine linkers.

An interesting methodology to determine the immunoreactivity of the PICs has been described by Stanaloudi *et al.*[86] scFv fragments were modified with a His-Tag to purify the PIC but also to label the mAb fragment for flow cytometry detection of the PIC. After blocking of unspecific binding sites, colon adenocarcinoma Caco-2 cells were incubated with scFv PICs, washed and incubated with an anti His-Tag mouse mAb which was detected by anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Figure 7). Stanaloudi *et al.*[86] also demonstrated that the use of a highly lipophilic PS promotes non-covalent interaction between protein and PS and binding in the mAb recognition site.

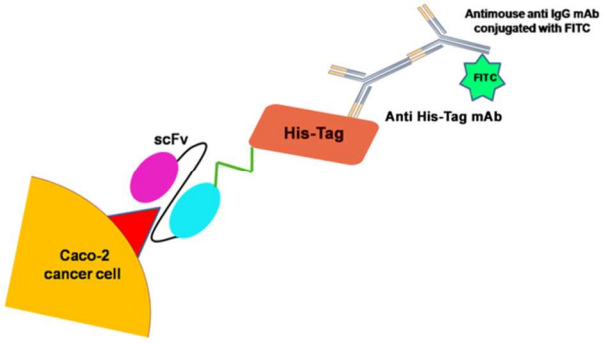


Fig. 7 Methodology used by Stanaloudi *et al.*[86] to determine PICs immunoreactivity.

6.1.2. Cellular accumulation of photo-immunoconjugates

After PIC binding to the targeted antigen it can stay attached to the plasma membrane or it can be internalized. The cellular localization of PICs depends on mAb size and type of antigen which is recognized by the PIC. Whole mAb conjugates are rather retained in plasma membranes due to their high molecular weight (110-140 kDa) which limits their intracellular accumulation. On the other hand, conjugates with mAb fragments (scFv, Fab') most probably are internalized due to receptor mediated endocytosis. Cellular localization of PICs has significant impact on PDT efficiency and cellular cell death pathway following PDT. The cellular localization of PICs can also be influenced by the temperature of incubation, side chain modifications of PIC and hydrophobicity/hydrophilicity (amphiphilicity) of conjugated PSs.

Methods of determination of the intracellular accumulation of the PIC are based on comparison of fluorescence or radioactivity

measurements of PIC extracted from cell or cell membrane with a calibration curve of standard solutions of PICs or free PS standards. Hamblin *et al.* reported that the intracellular accumulation is active for both cationic and anionic PICs, since it was lower at 4ºC than at 37ºC.[56] Additionally, cationic and anionic PICs have significantly different cellular accumulation profiles. The intracellular accumulation of the cationic PIC was 17 times higher than that of free PS and 12 times higher than with an anionic PS. The explanation of the aforementioned results is connected with better cellular binding and accumulation of cationic PICs as the result of overall net negative charge of the external cell membrane of malignant cells and its charge interaction with cationic modified PICs.[53,57] Cancer cells have even more negative charge in comparison with non-malignant cells due to superficial overexpression of the anionic carbohydrate polysialic acid. Several studies have indicated that (although not a requirement for potency) the conjugation of PSs with internalizing mAbs improves the photodynamic activity when compared with PSs conjugated with non-internalizing mAbs.[38,46,47,50,73]

6.1.3. Phototoxicity of photo-immunoconjugates

Ideally a PIC should be cytotoxic only after light activation and be active only after binding to target cancer cells. In case of PICs containing clinically used mAbs this requirement can be difficult due to their own cytotoxicity. Mitsunaga *et al.* have demonstrated that the unconjugated and clinically registered panitumumab, has significant cytotoxicity in human vulvar epidermoid carcinoma cells as effect of human epidermal growth factor receptor-1 downregulation and signal inhibition.[21] However, cytotoxicity results of PDT with PICs certainly have shown that the same effect was obtained with significantly lower concentrations of PICs in comparison with unconjugated PSs.

The photocytotoxic efficiency of PICs has been correlated with their ability to target cancer cells expressing the respective antigen. As the photodynamic effect is dependent on the generation of ROS (mainly $^1O_2$, which has a short lifetime in cells and limited migration), the site of primary ROS generation determines which structures may be destroyed after PDT. PIC cellular localization is therefore a key factor as it determines cellular primary localization damage and cell death mechanism following PIT. Studies performed with the clinically used trastuzumab conjugated with a phthalocyanine have demonstrated that cell death induced after photodynamic activation was dependent on the specific cell membrane binding of PIC and it was not dependent on its intracellular localization.[21] Other studies have demonstrated that PSs conjugated to internalizing mAbs[38,39,46,47,50,72,73] are able to produce higher *in vitro* photocytotoxic effects than PSs conjugated with non-internalizing mAbs. Studies performed by Vrouenraets *et al.* indicated that the phototoxicity of PICs is related with their total binding (*i.e.* internalized and surface bound conjugates).[48]

The photodynamic activity of cationic and anionic polylysine PICs have demonstrated that the cationic charge has an additive effect on the phototoxicity, probably due to the higher accumulation of these PICs inside of cancer cells.[56] The promising results obtained with cationic PICs, prompted Duska *et al.* to perform *ex vivo* studies with cationic PICs in combination with the chemotherapeutic cisplatin.[55] This treatment resulted in an increase of cytotoxicity when compared with the toxicity induced by cisplatin.

Not only properties of mAb but also chemical modification of side chains have great influence on PIC´s properties and thereby on PDT efficiency. The use of polyethylene glycol (PEG) chains to create a distance between hydrophobic PS and mAb is an attractive bioconjugation strategy to obtain PICs with high photodynamic activity.[44,83,84]

Figure 8 highlights the main *in vitro* and *in vivo* experiments that must be done with PICs.

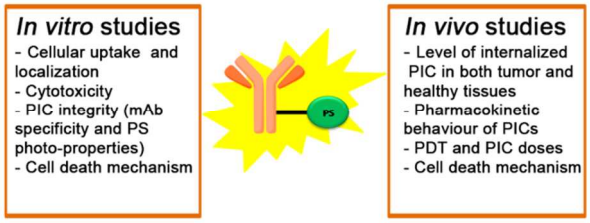


Fig. 8 Biological studies necessary during *in vitro* and *in vivo* validation of the photodynamic efficacy of PICs.

6.2. *In vivo* studies with photo-immunoconjugates

The promising results of *in vitro* anticancer activity of PICs have motivated the study of their *in vivo* efficacy. The biodistribution of PICs has been determined by fluorescence imaging of whole animal´s body or fluorescence *ex vivo* (in various organs after scarify the animal),[41,87] fluorescence spectroscopy[53,55,61,80,88] or by use of radiolabeled PICs.[46,47,50,64] The therapeutic efficacy of PIT has been evaluated in mice by determining the tumor volume using specific external calipers[21] or by noninvasive bioluminescence imaging.[74]

The biodistribution studies have demonstrated that after intravenous injection, PICs highly localize in normal tissues (namely on tissues of the reticulo endothelial system).[46] On the other hand, intratumoral administration (*i.e.* direct injection of the PIC in the tumor) results in longer retention in cancer tissues and reduced accumulation in normal cells.[89]

The doses of mAb and light irradiation seem to have an important role in PDT efficacy, since tumor recurrences have been observed in animals treated with a single dose of light irradiation.[21] The fractioned administrations of PIC at low doses and repeated exposures of light could result in complete tumor eradication.[61,63,71,90]

PICs have demonstrated longer term growth inhibition than the clinically used PS Photofrin®.[45,88] Additionally PICs have demonstrated higher tumor selectivity when compared with the respective non-conjugated PS.

Considering the aforementioned *in vitro* results with cationic and anionic PICs, *in vivo* studies to test the hypothesis of PIC charge influence on the PIT effect were performed.[54] Biodistribution studies demonstrated that cationic PICs showed highest tumor accumulation and phototoxicity.[54,58] However, Duska *et al.* reported separation of antibody and PS post intraperitoneal injection, which was higher for cationic than for anionic PICs.[54] Other studies have demonstrated that anionic PICs induce stronger phototoxicity than cationic PICs after intravenous administration.[53,59] Combining these contradictory results it was supposed

that the administration route has influence on PIC/cancer cell interaction. Polyanionic PICs are more effective when administered intravenously and polycationic PICs perform better after intraperitoneal injection.

The *in vivo* studies with PICs have also compared several formats of antibodies, demonstrating that the SIP format is highly stable *in vivo*, has high selectivity for tumors and is characterized by prolonged accumulation in cancer tissues.[35,90] *In vivo* SIP PICs have shown a high ability of targeting and disruption of tumor blood vessels followed by extensive hemorrhage and oedema of tumor.[35] Also PICs containing PS and scFv fragments have demonstrated their ability to target tumor vasculature and to induce thrombosis of tumor vessels after PDT.[90] However, PDT with PSs conjugated to scFv fragments resulted in tumor re-growth.

7. Conclusions

Most of the bioconjugation strategies applied in the development of PICs are based on PSs that have previously demonstrated promising anti-tumor activities as single PSs. In general, both mAb immunospecificity and PS photodynamic activity are retained after conjugation. The enhanced photodynamic activity of PICs is related to the capacity of the antibody to recognize antigens overexpressed in cancer cells and weakly expressed in healthy tissues. Unfortunately, PICs are not being studied in clinical cancer therapy. No studies have been reported to specify which PSs are best suited in the preparation of PICs. Most of the published studies are based on synthetic methodologies for the preparation of new PICs, rather than on biologic studies to assess the intracellular mechanisms of internalization and action of antibody-targeted PDT which is of utmost importance in the development of more efficient PICs.

In the development of PICs there are several issues which need to be considered such as:1) the evaluation of the optimal chemical strategies (direct or indirect) and functionalities to be used, 2) the assessment of mAb specificity and PS photo–chemical and –physical properties, 3) the determination of optimal whole antibody or antibody fragment to be used. Furthermore, some key points need to be taken into account on biological studies with PICs:

1) the internalization mechanism of PIC,
2) the type of cell death mechanism induced in tumor tissues after PDT with PICs (apoptosis, necrosis and autophagy) and their effects on neighbouring healthy tissues,
3) the factors influencing subcellular localization of a PIC (*e.g.* the chemical nature of PS, the immunoreactivity of the mAb, phenotype of the target cell),
4) the level of internalized PIC in both tumor and adjacent healthy tissues,
5) the time of light delivery,
6) the pharmacokinetic behaviour of PICs including their distribution through the body and its specificity for target tumor, metabolism and excretion.

The data summarized herein show the various chemical strategies applied in the synthesis of PICs as well as the most promising biological results obtained with these conjugates. This review has focused on the potentiality of PICs for the treatment of tumors. Nevertheless, it is important to point out that PICs are also being studied as fluorescent probes in the detection of tumors, in the treatment of infectious disease[91] and in the elimination of a specific cell population from a mixture.[92]

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Notes and references

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