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ARTICLE TYPE

Armed-antibody 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

10 photo-immunoconjugates (PICs), which keep the dual chemical and biological functionalities: photoproperties 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

15 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-

- 20 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
- 25 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
- 30 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

- 35 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
- 40 engineering have contributed to the development of more efficient PICs.¹⁻³ From a clinical point of view, 70 photoimmunotherapy is a promising approach in cases where the desired selectivity utilizing controlled precise application of light to diseased tissue might not be possible
- 45 (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 PICs¹¹⁻¹³ and there are no ongoing clinical studies using PICs. Ideally, a PIC should Molecular targeted photodynamic therapy (PDT) relies on 50 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 self" molecular configurations which are conventionally 55 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. 60 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 65 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

- 5 anatomical site being treated.^{4,14} First, PSs are preferentially 45 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 $({}^{1}O_{2})$ which has been shown to
- 10 be the main type of ROS responsible for tumor destruction.^{1,9,15} Nowadays, the discovery of new PSs with outstanding photo-50 **3. Molecular properties of antibodies** 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
- 15 generation time: first, second and third generation PSs (Figure $2),^{4,18}$

The first generation PSs are based on hematoporphyrin and its 55 derivatives. The second generation PSs include benzoporphyrin derivates, chlorins, phthalocyanines, texaphyrins and natural

- 20 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 60electromagnetic spectrum (600-800 nm), allowing the treatment of deep tumors due to deeper tissue penetration by red light
- 25 (Figure 2). Furthermore, with second generation PSs, the $^{1}O_{2}$ production is markedly improved at these wavelengths compared to the first generation PSs. Knowing that first and second 65 generation PSs are non-selective for cancer cells and can also cause toxicity in healthy cells, third generation PSs were
- 30 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



35 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

- 40 third generation PSs. Although liposomes and nanoparticles can improve tumor accumulation, the biomolecules with specificity for structural features overexpressed or enhanced in tumorassociated 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

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 $V_{\rm L}$ and $V_{\rm H}$, respectively (Figure 3). On the other hand, the Cterminal 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 $V_{\rm L}$ and the variable portion of the $V_{\rm H}$ 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^I) 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 80 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 85 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 effect. Nowadays, several reports have suggested that high DOLs may disturb the immunoreactivity of the mAb

- 5 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
- 10 immune system, resulting in immunogenic response induction. 55 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

- 15 clearance. While mAb fragments have advantages over whole 60 the bioconjugation steps in darkness and with all the solvents 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
- 20 production of PICs are single-chain variable fragments (scFv, 65 resulting PICs is determined after its purification by spectroscopy 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
- 25 in terms of blood clearance is intermediate between antibody 70 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
- 30 camelid species. They have a unique structure that is devoid of 75 purification and assessment of PIC integrity the next steps consist 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
- 35 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-40 immunoconjugates

During the development of PICs there are several combinations overcome the limited success of PIT (especially when applied in vivo) it is important to choose the appropriate type of mAb,

45 synthetic route and type of PS modification. The PS should

exhibit outstanding photophysical properties, such as photostability, high ability to generate ¹O₂ and absorption bands in the red and near-infrared regions of the electromagnetic spectrum (Figure 2). The PS also needs to have a reactive

- 50 chemical group/linking group for direct or indirect coupling to the amine,^{10,35,36,38-52} sulfhydryl^{33,53-59} or aldehyde⁶⁰⁻⁶³ 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 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 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
- 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
- $80\ \text{validation}$ 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
- 85 assays of antigen immobilized on sepharose matrix.⁶⁴ The photophysical properties of the conjugated PS, such as solubility, generation of ${}^{1}O_{2}$ and photo-stability can be determined by spectroscopic methods.

In vitro and in vivo evaluation of PICs is of paramount 90 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.

95 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 of PSs and mAbs that can be tested (Figure 5). In order to100 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 photo-5 immunoconjugates 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 esters, 22,35,36,42,46-50,64,70-74 activated amination.69

- 10 isothiocyanate, ^{43,45,75,76} maleimide³³, acryloyl functionalities⁷⁷ or by classical click-type reactions (copper-catalysed azide-alkyne cycloaddition).78 The indirect coupling implies the preconjugation of PSs with carriers or scaffolds such as dextran,79-81 polylysine,56,58,59 N-(2-hydroxypropyl)methacrylamide,⁸²
- 15 polyvinyl alcohol^{40,41} or polyglutamic acid.^{60,62} The indirect coupling improves PS solubility and does not affect its photophysical efficiency after conjugation. The indirect conjugation can also be performed by pre-coupling of mAb onto polyethylene glycol linker.⁸³ The coupling of photosensitizers
- 20 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
- 25 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
- 30 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-

- 35 hydrochloric acid (EDCI), that reacts with carboxyl groups of the PS (Scheme 1) to produce the key intermediate (Oacvlisourea).^{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 65 Bhatti et al. reported the coupling of verteporfin succinimidyl
- 40 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 noncovalently bound PS in the PIC even after purification.¹⁰



antibodies (*means representation of a possible conjugate).

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

50 The problems associated with the carbodilimide strategy led to the development of PSs containing activated esters. With this method, the carboxy groups of the PS are transformed into Nhydroxysuccinimide esters after treatment with N_{-} hydroxysuccinimide (NHS) in the presence of N,N'-55 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 60 reaction with the addition of NHS ester (Scheme 2).



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

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 70 of lysines have implications on the design of new PICs.49 Recently, the NHS ester of the commercially available silicon phthalocyanine derivative (described as the near-infrared 60 was observed. The synthetic procedures developed by fluorescent PS, IRDye700DX) has been conjugated with whole antibodies (trastuzumab,²¹ panitumumab²¹ and anti-human carcinoembryonic antigen⁷⁴) or with nanobodies⁷² targeting

- 5 epidermal growth factor receptors. After conjugation, PICs retained the immunoreactivity of the antibody and the DOL was 65lower 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
- 10 phthalocyanine (AlPcS₄) with whole mAbs overexpressed in cancers. The first step in the synthetic procedure was the conversion of the AlPcS₄ sulfonic acid groups into sulfonyl chloride functionalities after treatment with thionvl
- 15 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
- 20 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 75 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,
- 25 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

- 30 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 SnChle6. Palumbo et al.35 also synthesized SIP PICs in an attempt to obtain a selective destruction of tumor neovasculature. 5-(4-Carboxyphenyl)-
- 35 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
- 40 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
- 45 5.2.1. Conjugations using esterification of carboxylic acid groups on PS to tetrafluorophenyl esters Vrouenraets et al. have developed bioconjugation strategies to

(mTHPC) with mAbs.⁴⁶ The commercially available mTHPC

- 50 (Foscan, Temoporfin) was radiolabeled and then tetracarboxymethylated using iodoacetic acid. Methylation increased mTHPC hydrophilicity and resulted in the formation of functional groups suitable for conversion into activated esters. The four carboxylic 105 immunoconjugates⁷⁵ acid groups were esterified using 2,3,5,6-tetrafluorophenol and
- 55 EDCI (Scheme 3). The conjugation of the 131 I-labeled PS with the respective 125I-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 ¹²⁵I:¹³¹I molar ratio, however formation of mAb aggregates

- 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(SO₃H)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-\phiCO2H),47 a porphyrin derivative more foetal colon and colon adenocarcinomas, breast and ovarian 70 hydrophilic than mTHPC 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 80 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 $85 al.^{43}$ 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 90 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 95 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 tri-cationic porphyrins bearing an isothiocyanate group with mAbs. The capability of porphyrins couple the poorly water soluble *meta*-tetrahydroxyphenylchlorin100 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 phthalocyaninewas 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

- 5 In this methodology, maleimide substituted PSs are conjugated 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
- 10 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
- 15 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
- 20 ester group were reacted with 1,6-diaminohexane or O,O'-di-(2aminoethyl)-hexaethylene glycol which had one amino group 60 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
- 25 crosslinker succinimidyl-4-(N-maleimido-methyl)cyclohexane-1carboxylate 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
- 30 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 with a small hydrocarbon spacer and porphyrin with a long
- 35 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.

40

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 80 verteporfin with polyethylene glycol resulted in lower uptake and

- motivated its use in the development of PICs. The most well 45 known example of a click reaction is the copper-catalyzed azidealkyne cylcoaddition, which yields a 1,4-disubstituted fivemembered 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 50 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 with mAbs containing a sulfhydryl reactive group, forming a 55 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 65 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 corresponding to porphyrin linked directly to SIP(L19), porphyrin 70 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 75 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) PIC demonstrated accumulation in lysosomes and endosomes⁸⁴ while free hydrophobic BPD verteporfin was accumulated in lipophilic compartments of the cells like mitochondria and

- 5 perinuclear region. The same PIC was also evaluated by Abutransport 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}-
- 10 monoethylenediamine monoamide derivative (Chl_{e6}-MA) with the carbohydrate moieties (at the hinge region) of several 60antibodies (Scheme 7).^{60,62} Herein, the mAb previously oxidized by reaction in sodium periodate is added to the functionalized Chle6-MA-polyglutamic acid bearing amino groups. The
- 15 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 noncovalently bound Chle6-MA, as well as some evidence of crosslinking.



the hinge region) of antibodies via polyglutamic acid linker functionalized with hydrazine.

6. Photo-immunoconjugates as photodynamic 25 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

30 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}

35 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

- 40 reactive groups of the PSs. While whole mAbs contain more lysine residues than mAb fragments, the possibility 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
- 45 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

- phototoxicity when compared with free BPD verteporfin.⁴⁴ The 50 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
- Yousif et al. who demonstrated that it undergoes vesicle mediated 55 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}
 - 65 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.
 - 70 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
 - 75 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-
 - 80 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.

85 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. Efficiency in internalization The cellular localization of PICs depends on mAb size and type of antigen which is recognized by the PIC. Whole 90 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 95 has significant impact on PDT efficiency and cellular cell death

pathway following PDT. The cellular internalization localization

of PICs can also be influenced by the temperature of incubation, side chain modifications of PIC hydrophobicity/hydrophilicity (amphiphilicity) of conjugated PSs.

- 5 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 65 calibration curve of standard solutions of PICs or free PS standards. Hamblin et al. reported that the intracellular
- 10 accumulation is active for both cationic and anionic PICs, since it was lower at 4°C than at 37°C.56 Additionally, cationic and profiles. The intracellular accumulation of the cationic PIC was 17 times higher than that of free PS and 12 times higher than with
- 15 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 75 external cell membrane of malignant cells and its charge interaction with cationic modified PICs.53,57 Cancer cells have
- 20 even more negative charge in comparison with non-malignant cells due to superficial overexpression of the anionic carbohydrate polysialic acid. The fact Several studies have indicated that (although not a requirement for potency) the conjugation of PSs conjugated with internalizing mAbs have
- 25 better accumulation improves the photodynamic activity than when compared with PSs conjugated with non-internalizing mAbs.38,46,47,50,73

6.1.3. Phototoxicity of photo-immunoconjugates

- 30 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
- 35 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 40 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 1O2, which has a short lifetime in cells
- 45 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
- 50 trastuzumab conjugated with a phthalocyanine have demonstrated 100that 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
- 55 mAbs^{38,39,46,47,50,72,73} are able to produce higher *in vitro*105 photocytotoxic effects than PSs conjugated with non-internalizing mAbs, due to their high uptake. Studies performed by Vrouenraets et al. indicated that the phototoxicity of PICs is

related with their total binding (i.e. internalized and surface and 60 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.
- anionic PICs have significantly different cellular accumulation 70 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

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The promising results of in vitro anticancer activity of PICs have motivated the study of their in vivo efficacy. The biodistribution 85 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 90 using specific external calipers²¹ 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 95 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 60 accumulation and phototoxicity.54,58 However, Duska et al.

- 5 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
- 10 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 15 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 20 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 regrowth.

25 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 85 mAb immunospecificity and PS photodynamic activity are

- 30 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
- 35 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 40 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 -
- 45 physical properties, 3) the determination of optimal whole 100 Berlin, Germany 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,

- 50 2) the type of cell death mechanism induced in tumor tissues after 105 Coimbra, 3000-548 Coimbra, Portugal 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,

55 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

65 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 disease91 and in the elimination of a specific cell population from a mixture.92

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