Site-specific chelator-antibody conjugation for PET and SPECT imaging with radiometals

Mauricio Morais*, Michelle T. Ma

School of Biomedical Engineering and Imaging Sciences, King’s College London, St. Thomas’ Hospital, London SE1 7EH, United Kingdom

Antibodies and their derivatives radiolabelled with positron- and gamma-emitting radiometals enable sensitive and quantitative molecular Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) imaging of antibody distribution in vivo. Chelators that are covalently attached to antibodies allow radiolabelling with metallic PET and SPECT radioisotopes. Conventional strategies for chelator-protein conjugation generate heterogeneous mixtures of bioconjugates that can exhibit reduced affinity for their receptor targets, and undesirable biodistribution and pharmacokinetics. Recent advances in bioconjugation technology enable site-specific modification to generate well-defined constructs with superior properties. Herein we survey existing site-specific chelator-protein conjugation methods. These include chelator attachment to cysteines/disulfide bonds or the glycans region of the antibody, enzyme-mediated chelator conjugation, and incorporation of sequences of amino acids that chelate the radiometal. Such technology will allow better use of PET and SPECT imaging in the development of antibody-based therapies.

Introduction

Monoclonal antibodies (mAbs) have demonstrated exquisite sensitivity and selectivity for their target cell surface receptors in vivo [1]. As well as being important in clinical therapies [2,3], mAbs can be used as in vivo vectors, to deliver an additional therapeutic payload (e.g. small-molecule cytotoxic compounds [4–6] or radiotherapeutic isotopes [7,8]) or, in combination with an imaging probe (e.g. a gamma or positron-emitting radionuclide, or an optically active molecule), to visualize the in vivo distribution of target cell surface receptors.

Antibodies labelled with a gamma- or positron-emitting radionuclide can be used to quantitatively image the biodistribution of the radiolabelled-antibody using whole body Single Photon Emission Computed Tomography (SPECT) or Positron Emission Tomography (PET) respectively. Such radiolabelled mAbs are extremely useful for both preclinical and clinical development of antibody-based therapies, enabling (i) non-invasive detection of the target receptors’ expression, including any potential heterogeneity in expression, (ii) estimation of an antibody’s biodistribution, therapeutic index and pharmacokinetics by quantification of antibody distribution in target and normal tissues, and (iii) prediction and assessment of a patient’s response to a specific mAb therapy by imaging with the radiolabelled antibody [9].
Radioactive metal ions are well-suited to radiolabelling antibodies for PET and SPECT imaging. Compared to non-metallic radionuclides, radiometals allow for simple radiolabelling procedures: typically, a chelator is firstly covalently attached to the antibody, and once conjugated, the chelator binds the radiometal. The half-lives of many of the metallic radionuclides, including zirconium-89 [9] (78 h half-life) for PET, and indium-111 [10] (67 h half-life) for SPECT, more closely match the time required for antibodies to clear circulation and accumulate in target tissue (1 day–1 week) than non-metallic radionuclides such as fluorine-18 (119 min half-life). Antibodies labelled with PET, SPECT and radiotherapeutic radioisotopes of iodine have been extensively studied both preclinically and clinically [11], however, many of these are subject to deiodination in vivo. Advances in radiochemical methodology have increased stability of radioiodine-antibody constructs [12,13], however, this is beyond the scope of this review.

**Antibody structure**

Immunoglobulin type 1 antibodies (Fig. 1) (IgGs) are the most commonly used type of mAb for pharmaceutical applications. They are approximately 150 kDa, and are composed of two identical polypeptide “heavy chains” paired with two light chains. Each antibody contains three domains: a constant region, a hinge region, and an antigen-binding region. The constant regions of both the heavy and light chains consist of a flexible hinge region that allows the antibody to flex and bind to antigens. The antigen-binding region contains the variable domain, which is responsible for specificity and affinity of the antibody.

**Fig. 1.** Structure of IgG1 antibodies and smaller, engineered fragment antibodies.
“light chains”. They include a fragment antigen-binding (Fab) region, a fragment crystallisable (Fc) region, two disulfide bonds in the hinge region and a conserved glycosylated position at N297 of each heavy chain [1,5]. Smaller derivatives of IgGs that include the targeting variable region of the Fab region have also been engineered. Although they generally exhibit lower accumulation at disease sites, they clear circulation faster than full-length IgGs [14]. Recently described radiolabelled-immunoconjugates include both full-length IgG mAbs, and smaller fragment derivatives [14,15]. Radionuclide imaging with these smaller derivatives has shown that high “target to non-target” contrast can be achieved at early time points (1–12 h) following radiotracer administration. In contrast, full-length IgG antibodies require significantly greater time periods (1 day–1 week) to enable the antibody to accumulate at target tissue and clear circulation.

**Chelators for radiometal-antibody imaging**

Metallic radioisotopes are incorporated into an antibody via a chelator. Many factors influence the choice of a metallic radioisotope, including the imaging modality (PET or SPECT/γ-scintigraphy imaging), matching of the half-life of the radioisotope to the pharmacokinetics of the vector, and the availability of the radioisotope itself. The chelator binds the radiometal, and the resulting radiometal–chelator complex will ideally possess both high thermodynamic and kinetic stability. This high stability is essential to ensure that the radiometal remains bound to the antibody in vivo. We [16–18] and others [19–21] have reviewed existing and new chelator technology for radiometal-based PET and SPECT imaging. Here, we simply include a list of commonly used imaging radiometals (Table 1) for ease of reference.

**Attaching chelators to antibodies**

In chelator-antibody conjugation reactions, the antibody and chelator contain complementary reactive functional groups for attachment to each other. There are several requirements for this covalent attachment:

(i) mild conjugation (and subsequent radiolabelling) reaction conditions are essential to preserve the tertiary and quaternary structure of the antibody;
(ii) the new covalent link between the chelator and protein must be stable under physiological conditions; and
(iii) the covalent modification must not compromise the binding affinity and specificity of the protein.

Most of the conventional functionalities used to attach chelators to proteins consist of reactive electrophilic groups such as isothiocyanates, N-hydroxysuccinimide esters (Fig. 2) and anhydrides that react with solvent accessible primary amines of lysine side chains, and maleimides that attach via Michael addition to the thiols of reduced cysteine side chains (Fig. 3a) [18,22–26].

In these conventional conjugation reactions, the presence of multiple solvent-accessible amino acids in proteins leads to a lack of both stoichiometric control and site-specificity. The resulting heterogeneous mixtures of chelator-protein conjugates can exhibit suboptimal pharmacokinetics and decreased affinity for target receptors [27–29]. Additionally, the heterogeneous nature of the conjugates is potentially a barrier to regulatory approval of their clinical application and development.

Significant efforts over the last decade have resulted in new site-selective conjugation methods for attaching chelators and other cargoes (fluorescent molecules, small molecular weight drugs) to antibodies [30–39]. Such an approach (often described as orthogonal) uses complementary pairs of functional groups that react chemoselectively with each other. It involves appending one functional group to the chelator, and the other to the protein, followed by reaction between the two motifs. Ideally, this reaction will proceed in water at near-neutral pH and ambient temperature (25–37 °C) to avoid protein denaturation or degradation.

“Click chemistry” is one such approach [40,41]: for example, engineering cyclooctyne and azide groups into a chelator and antibody to give a triazole-containing bioconjugate, can provide this desired chemoselectively. However, this approach requires modification of the antibody prior to the conjugation reaction itself, and if the azide/azide is incor-

---

**Table 1. Decay properties and production methods for selected radiometals used in PET and SPECT imaging.**

| Radiometal | Half-life | Mode of decay (%) | Production mode | Application |
|------------|----------|-------------------|-----------------|-------------|
| 99mTc      | 6.0 h    | IT⁺ (100)         | ⁹⁹Mo/⁹⁹mTc generator | SPECT      |
| ¹¹¹In      | 2.83 d   | EC⁺ (100)         | ¹¹¹Cd(p,n)¹¹¹In   | SPECT      |
| ⁶⁷Ga       | 3.27 d   | EC (100)          | ⁶⁸Zn(p,n)⁶⁷Ga     | SPECT      |
| ⁶⁸Ga       | 68 min   | β⁺⁻ (90)          | ⁶⁸Ge/⁶⁸Ga generator | PET        |
| ⁸⁹Zr       | 78.4 h   | EC (77) β⁺ (23)   | ⁹⁰Y(p,n)⁸⁹Zr      | PET        |
| ⁶⁴Cu       | 12.7 h   | β⁺⁻ (18) EC (43)  | ⁶⁴Ni(p,n)⁶⁴Cu     | PET        |

*1T = isomeric transition.
EC = electron capture.
β⁺⁻ = positron emission.
β = beta emission.
Cysteine modification

Cysteines are useful for selective protein modification for several reasons:

- Incorporation via a non-specific lysine modification, as is often the case, site-selectivity is not actually achieved [42–45]. There are elegant examples in which reactive “click” groups have been site-selectively introduced into antibodies [30,46], and examples are included in relevant sections below.

- Here, we capture an overview of site-selective conjugation methods used to prepare radiometal-labelled antibodies and antibody derivatives for PET and SPECT imaging. This includes site-directed modification of cysteines/disulfide bonds and the glycan region of the antibody, enzyme-mediated conjugation, and incorporation of sequences of amino acids that coordinate the radiometal. Others have also recently reviewed this area, including an elegant and detailed survey of site-selective antibody conjugation methods used for molecular imaging with both optical and radionuclide imaging labels, and site-selective methods to incorporate radiotherapeutic isotopes [47,48].

- **Cysteine modification**

Cysteines are useful for selective protein modification for several reasons:
(i) cysteine has a low abundance (1–2%) in living organisms [49,50], and so the probability of adversely affecting the pharmacokinetics of proteins by attachment of too many chelators is relatively low; (ii) the nucleophilicity of the cysteine deprotonated thiol group ($pK_a$ of $\sim 8.3$) exceeds the reactivity of other nucleophilic groups of amino acids in proteins [51,52]; (iii) single, solvent-exposed cysteine residues can be engineered into antibodies and their derivatives [53,54], enabling site-selective attachment of cargo, including radiolabelled chelators. In many cases, such cysteines are introduced at the carboxyl terminus of the protein, to minimise the likelihood that the modification will impair protein structure and activity. These new cysteine residues are often “capped” by a single cysteine amino acid or thiol-containing small molecule [55], or form protein homodimers via intermolecular disulfide bonds [56]. Thus, a reduction step is required to generate a reduced thiol for subsequent conjugation. Full-length antibodies [57] and other small proteins [58,59] have been modified using this approach.

**Conjugation via C–S bonds**

The most widely employed method of conjugating antibodies via cysteines involves a Michael addition reaction of a thiol

Fig. 3. Thiol-reactive compounds including (a) maleimide, (b) pyridyldithiopropionate, (c) methylsulfonyl phenyloxadiazole, (d) monobromo maleimide and (e) carboxyacrylic derivatives of chelators and fluorophores can be used to incorporate chelators into antibodies and their derivatives. Here we also illustrate incorporation of an $^{89}$Zr-DFO (desferrioxamine) chelator and an $^{111}$In-DTPA (diethylenetriamine pentaacetate) chelator. Metal complex charges are excluded.
with a maleimide to form a succinimidyld thioether adduct (Fig. 3a).

Radionuclide imaging studies have demonstrated that succinimidyld thioether linkages have superior stability compared to disulfide linkages. For example, maleimide and pyridyl-dithiopropionate (Fig. 3a/b) groups appended to DTPA chelators have been reacted with terminal cysteine residues of an anti-carcinoembryonic Fab’ [60]. The new DTPA-Fab’ conjugates were radiolabelled with $^{111}$In, and their biodistribution assessed in mice bearing colorectal cancer tumours. The (maleimide-derived) thioether-linked radiotracer enabled efficient tumour targeting, whereas the (pyridyl-dithiopropionate-derived) disulfide-bridged analogue showed poor biodistribution with high kidney uptake and poor tumour targeting, due to in vivo cleavage of the S–S linkage.

Maleimide derivatives have been widely used to incorporate chelators, via cysteine thiols, into antibody derivatives and proteins [26,55–59], and many chelator-maleimide reagents are commercially available. However, maleimide conjugates suffer from instability: the thioether can undergo a retro-Michael reaction, converting back to the starting thiol and maleimide. The maleimide motif, still attached to its payload, reacts with endogenous molecules containing bioavailable thiols, such as glutathione and albumin [61–64]. In radionuclide imaging, this can potentially result in accumulation of radioactivity at off-target sites, decreasing image contrast, sensitivity and the ability to quantify protein biodistribution.

Several new cysteine-reactive reagents that provide enhanced conjugate stability have been developed [64–67]. Following reduction of antibody disulfide bonds (typically with tris(2-carboxyethyl)phosphine hydrochloride), methylsulfonyl phenyloxadiazole (Fig. 3c) derivatives bearing DFO and DTPA chelators have been selectively attached to cysteines of trastuzumab, cetuximab and huA33 antibodies [68,69]. These chelator-antibody conjugates contained an average of 1.4–2.2 chelators per antibody and gave $^{89}$Zr-DFO-mAbs and radiotherapeutic $^{177}$Lu-DTPA-mAbs that demonstrated higher C–S bond stability in serum than maleimide derivatives. Furthermore, methylsulfonyl phenyloxadiazole-derived $^{89}$Zr-DFO-huA33 antibody demonstrated superior in vivo targeting behaviour compared to its maleimide-derived analogue: the former resulted in higher tumour-to-background activity ratios in a murine model bearing huA33 antigen-expressing colorectal cancer xenografts.

Monobromo maleimide (Fig. 3d) [67,70] and carbonylacrylic (Fig. 3e) [71] reagents have been used to generate stable protein conjugates via native, single accessible cysteines, although to date, they have not been used in radionuclide imaging. Monobromo maleimides enable substitution of the bromo group by a reactive thiol, generating a thiol-maleimide that can be hydrolysed (ring-opened) to give a C–S bond that is stable to undesirable retro-Michael deconjugation [67,70].

**Modifying antibody disulfide bridges**

Single cysteine-targeted conjugation strategies are well-suited for site-specific modification of Fab or scFv fragments that contain single, exposed cysteine residues, however, they are not ideal for generating well-defined conjugates of IgG antibodies. IgG proteins contain four solvent-accessible interchain disulfide bridges in the protein hinge region, and their reduction creates eight reactive thiols. Conjugation to these reduced species results in heterogeneous mixtures of conjugates, and the cleavage and modification of these disulfide bridges (with up to eight copies of cargo) can lead to adverse pharmacokinetics, and reduce the metabolic stability of IgG antibodies in plasma [72,73]. Additionally, reduced thiol groups that do not participate in bioconjugate reactions can undergo oxidative intramolecular reactions with other thiols, often resulting in disulfide scrambling that disrupts the structure and function of the protein.

Recent research has produced functional species that react with two reduced thiol groups of antibodies, thus enabling concomitant attachment of functional groups (for example, fluorophores) and re-bridging of two cysteines. It is important that the re-bridging motif reacts rapidly with both disulfide-derived reduced thiols, to avoid incorrect re-bridging, and thus preserve structure and function of the protein [74–76], and that the resulting re-bridged covalent bonds are unreactive towards serum thiols. Novel thiol-stable chemical technologies have been successfully applied to modification of disulfide bonds in mAbs and their derivatives. These include bisulfone derivatives (Fig. 4a) [77–80], dibromoalkyl oxetane derivatives [81] (Fig. 4b), trivalent arsenous acid [82], dibromopyridazines (Fig. 4c) [73,83], and disubstituted maleimides (Fig. 4d) [84–88]. Bridged bisthiomaleimide (derived from dissubstituted maleimides) can be hydrolysed to dithiomaleamic acid under mildly basic conditions, generating homogenous antibody conjugates that are unreactive towards serum thiols and do not undergo retro-Michael reactions in biological media, unlike conventional maleimide derivatives [85,89,90]. These technologies have not been applied to radionuclide imaging, but could be advantageous for future antibody-based radionuclide imaging.

**Glycan modification**

IgG proteins contain two conserved post-translational modification glycosylation sites (Fig. 1) that can be chemically modified to enable site-selective attachment of chelators. This conjugation strategy is appealing because:

(i) modification at these sites will not compromise antigen binding properties as they are distal to the Fab region;
(ii) there are two attachment sites available per antibody; and
(iii) there are several chemoselective/orthogonal reactions enabling modification of glycans and hexose groups.

This method is not suitable for smaller antibody fragments that lack glycans, or for IgG1 antibodies whose function requires the presence of the native (unmodified) Fc regions for binding to Fc-receptors (such as those involved in immune responses).

**Importance of glycan modification chemistry to clinical antibody imaging**

Glycan-based modification chemistry has been critically important in the clinical development of molecular imaging with antibodies in nuclear medicine [91]. The first FDA-approved imaging radioimmunoconjugate specifically incorporated $^{111}$In into the satumomab antibody via a DTPA chelator at the glycan region (see below). $^{111}$In-DTPA-satumomab targets a tumour-associated glycoprotein, TAG-72, expressed in several cancers, including colorectal and ovarian cancers [92]. Clinical $\gamma$-scintigraphy and SPECT imaging trials in over 1000 patients have demonstrated $^{111}$In-DTPA-satumomab's utility in detecting colorectal and ovarian cancer lesions, and in combination with other diagnostic tests, informing clinical decision-making, including treatment and surgery.

Another FDA-approved antibody imaging agent, $^{111}$In-capromab pendetide (also known as Prostascint), similarly incorporates a DTPA chelator using this glycan technology [91]. $^{111}$In-capromab pendetide targets the prostate specific membrane antigen (PSMA) expressed in prostate cancer, and has demonstrated potential in assisting in (i) the staging of prostate cancer, particularly in identifying soft tissue metastases, and (ii) locating prostate cancer tumours when diagnostic blood tests indicate disease recurrence [93]. Although $^{111}$In-capromab pendetide has not demonstrated high positive predictive value and specificity for clinical management of prostate cancer, it has been fundamentally important in the development of radionuclide molecular PET and SPECT imaging of its target, PSMA. Several PSMA-targeted PET and SPECT imaging agents are currently being clinically developed [94–97], after showing high diagnostic utility in prostate cancer management.

**Reaction with oxidised hexose groups**

One of the most widely used methods for site-specific modification of glycan sites relies on the generation of an aldehyde

---

**Fig. 4.** New conjugation technology that enables disulfide rebridging includes derivatives based on (a) bissulfone, (b) dibromoalkyl oxetane, (c) dibromopyridazinediones and (d) dibromo/dithiophenyl maleimides. This technology has not been applied to radionuclide-labelling of antibodies, but is a promising future avenue.
group by oxidising the cis-glycol groups of terminal hexoses (Fig. 5a–c), commonly with sodium periodate [33,98–100]. The generated aldehydes can be reacted with chelator derivatives containing pendant primary amines, generating an imine conjugate. The resulting imine conjugate can be further modified by reduction with cyanoborohydride to form an amine, preventing in vivo hydrolysis of the newly generated linker (Fig. 5a) [101]. \(^{111}\text{In-DOTA}\)-satumomab and \(^{111}\text{In-capromab pendetide}\) both incorporate DTPA chelators via this strategy [91]. Compared to antibodies in which the radiolabel is incorporated via solvent-accessible lysines, radiolabelling via site-specific glycan modification leads to increased radioactivity accumulation in tumours and decreased off-target tissue radioactivity concentration [91,102].

Aldehydes of modified glycans can also be reacted with hydrazides (Fig. 5b), and in some cases the resulting hydrazide biocjugates have been reduced to generate more stable chelator-antibody derivatives [103–106]. A prostate-targeting hydrazide-linked chelator-antibody (CYT-351) labelled with \(^{99m}\text{Tc}\) (Fig. 5c) has enabled planar and SPECT imaging of prostate cancer in patients [103]. Glycan-derived aldehydes and ketones have been reacted with O-alkyl hydroxylamines to form oxime ethers (Fig. 5d). This covalent attachment is more stable than imine or hydrazine linkers and does not require subsequent reduction steps [107]. This method has been used to prepare a \(^{111}\text{In-DOTA}\)-trastuzumab species, which demonstrates conserved immuno-noreactivity for target HER2 receptors both in vitro and in tumour bearing mice [108].

**Enzymatic modification of glycans**

Although full-length antibodies have been successfully modified via glycan oxidation/conjugation, in some cases, the harsh oxidation conditions can lead to inadvertent oxidation of methionine residues of the antibody, reducing the serum half-life of the antibody [109]. A dual enzyme approach that enables functionalisation of glycans can avoid this: the enzyme \(\beta\)-1,4-galactosidase removes the terminal galactose residues of antibodies, and following this, the mutant enzyme \(\beta\)-1,4-galactosyltransferase (Y289L) incorporates a modified galactose unit containing a reactive functional group at this site [30,110]. This technology has been applied to site-directed radiolabelling of a J591 prostate-cancer targeting IgG [30], where the terminal galactose residue was substituted for a galactose motif containing an azide. In a copper-free azide/dibenzocyclooctyne cycloaddition “click” reaction, the DFO chelator was then conjugated to the antibody and radiolabelled to give \(^{89}\text{Zr-DFO-J591}\) IgG (Fig. 6a). This derivative showed higher tumour uptake than the randomly modified analogue (via lysine modification with isothiocyanate). Using this strategy, dual fluorescent, \(^{89}\text{Zr}\)-radiolabelled antibodies were prepared and showed efficacy in imaging A33 transmembrane glycoprotein expression in a colorectal tumour mouse model.

![Fig. 5. Oxidation of antibody glycan sites generates reactive aldehydes that selectively react with: (a) amines, (b/c) hydrazines and (d) hydroxylamines enabling site-specific attachment of chelators. Such methods have been used to incorporate a \(^{99m}\text{Tc}\)-tricine radiolabel and an \(^{111}\text{In-DOTA}\) (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate) radiolabel. Metal complex charges are excluded.](image-url)
Enzyme-mediated conjugation

Recently, protein technology has been developed to enable enzyme-mediated, site-specific conjugation of a cargo to target antibodies and proteins [111–114]. These methods make use of an enzyme that recognises two complementary motifs on the targeting antibody/protein and the cargo-containing compound. Using these two complementary motifs, the enzyme catalyses covalent attachment of the targeting antibody/protein to the cargo. The use of β-1,4-galactosyltransferase (Y289L) with DFO chelator (Section “Reaction with oxidised hexose groups”) is an example of this. Other examples of enzyme-mediated, site-specific conjugation of chelators to proteins also exist. Like reactions involving β-1,4-galactosyltransferase (Y289L), such reactions achieve chemoselective chelator conjugation under mild conditions that do not denature the antibody.

Transglutaminase

The bacterial transglutaminase (BTG) enzyme catalyses the formation of a new amide bond between the primary amine of a lysine side chain and the γ-carboxamide group of a glutamine side chain. Many 5-aminopentyl derivatives can act as lysine side chain surrogates and are recognized by BTG. However, BTG is only reactive towards glutamine side chains located in flexible regions of proteins/biomolecules. Glutamine side chains of IgG antibodies are thus normally unreactive to BTG. Removal of glycans from position N297 of an IgG antibody, using the enzyme N-glycosidase F (PNGaseF), results in increased flexibility of this antibody region. This gives rise to the increased BTG-catalysed reactivity of a glutamine residue, Q295, in close proximity to N297, and chelators bearing 5-aminopentyl groups can be site-selectively introduced into this position in the presence of BTG [115,116]. Reaction of either anti-L1-CAM chCE7 antibody or rituximab antibody (Fig. 6b), firstly with PNGaseF to remove N297 glycans, and secondly, with bifunctional chelators attached to 5-aminopentyl groups in the presence of BTG, results in IgG mAbs bearing only two chelators per antibody, attached at position Q295 [115]. Radionuclide imaging and biodistribution studies in tumour-bearing mice showed that this site-specific conjugation strategy led to radiolabelled antibodies that provided higher tumour to non-target organ contrast, compared to antibodies radiolabelled using conventional, non-specific methods (lysine modification).

Sortase A

Bacterial enzyme sortase A (SrtA) catalyses a transpeptidase reaction between an N-terminal glycine, and a specific amino
acid sequence, -Lys-Pro-X-Thr-Gly- (where X is any amino acid) [117–119]. SrT A (i) recognizes and cleaves this peptide sequence between threonine and glycine, and (ii) catalyses the formation of a new amide bond between threonine and an N-terminal glycine-containing species. Using this protein chemistry, a macrobicyclic sarcophagine (a 3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane derivative) that binds 64Cu has been site-selectively appended to a scFv that targets GPIIb/IIIa glycoprotein receptors overexpressed in thrombosis, athero-
sclerosis and inflammation. Two different conjugation strategies have been described. In the first SrT A-catalysed conjugation, the scFv, engineered to contain a -Lys-Pro-X-Thr-Gly- sequence at the C-terminus, was reacted with an N-terminal glycine residue attached to a sarcophagine chelator (Fig. 6c) [119]. In the second, the same scFv (in the presence of SrT A) was reacted with an N-terminal glycine attached to a strained cyclooctyne [118]. Once purified, the new scFv-cyclooctyne bioconjugate was reacted chemoselectively with an azide appended to a sarcophagine ligand. Whilst the first approach required fewer derivatisations to incorporate a chelator, the second approach yielded a scFv-octynyl bioconjugate that is more versatile — it can site-selectively incorporate any label bearing an azide motif. Both 64Cu-sarcophagine labelled scFv conjugates enabled PET imaging of GPIIb/IIIa glycoprotein receptors expressed in thrombosis.

Amino acid coordinating sequences

Peptides and proteins can bind to metal ions via amino acid side chains, carboxylate groups of C-termini, amine groups of N-termini, and nitrogen atoms of amide groups of the peptide backbone. Particular sequences of amino acids, containing several metal-binding ligands, enable direct radiometal complexation by the protein, without the requirement of a synthetic chelator. In such cases, the radiometal-binding amino acid sequence is simply engineered into the protein at the desired location.

His tags

The hexahistidine sequence, or His$_6$ tag, originally developed to aid in protein purification, has been prevalently applied to incorporate the SPECT isotope, 99mTc (in the form of 99mTc (CO)$_3$), into antibody derivatives [120–126]. The His$_6$ tag is commonly incorporated at the C-terminus of targeting proteins, although N-terminal incorporation is also possible. Many 99mTc-labelled proteins have been radiolabelled using this strategy, with recent examples including a HER2-targeted sdAb [123], a αβ-integrin-targeted diabody [121] and a PSMA-targeted diabody [122]. SPECT imaging with these radiotracers enables visualisation of target tumour tissue. Computational modelling suggests 99mTc(CO)$_3$ coordinates via two imidazole groups of a His$_6$ tag [127]. Modifications to the His$_6$ tag have improved protein radiolabelling and biodistribution. For example, the inclusion of a thiol-containing cysteine residue to a His$_6$-containing sequence can increase mettallolabelling efficiency [128]. Alternatively, substitution of His residues in the His$_6$ amino acid sequence can increase hydrophilicity and negative charge of a radiolabelled protein, which, in turn, can decrease radioactivity retention in non-target tissue in vivo, leading to improved image contrast. For example, substituting the sequence HHHHHH for HEHEHE does not adversely affect 99mTc-radiolabelling of a HER2-targeted protein, but it does decrease radioactivity retention in non-target tissue in vivo [129].

Sequences incorporating amide-binding motifs

Several low molecular weight 99mTc radiotracers, including 99mTc-labelled compounds used in renal imaging and 99mTc-labelled peptides that target cell surface receptors [19,96,130,131], make use of deprotonated amide groups of a peptide backbone in combination with deprotonated thiolis to coordinate 99mTc. Peptide sequences incorporating these features can be engineered into proteins for efficient binding of the 99mTc(CO)$_3$ motif. The first example of this demonstrated that Gly$_3$Cys, engineered into the C-terminus of a scFv protein, could be applied to SPECT imaging of a scFv disease target [132–136]. Subsequent studies have demonstrated that a scFv protein incorporating a C-terminal 99mTc(CO)$_3$-Gly$_3$Cys sequence has more favourable biodistribution properties (faster clearance and lower off-target organ retention) than a C-terminal 99mTc(CO)$_3$-His$_6$ homologue [136]. Glycine residues can be substituted for other amino acid residues without compromising 99mTc(CO)$_3$(O) binding abilities [96,134,135,137], however incorporation of the amino acid binding sequence Cys-Gly$_3$ directly at the N-terminus (instead of Gly$_3$Cys directly at the C-terminus) can lead to release of 99mTc radionuclide in vivo, compromising imaging ability [133,134].

Summary and concluding remarks

Advances in conjugation chemistry and protein engineering have enabled development of homogenous radiolabelled antibodies. New site-specific antibody modification strategies that have not yet been applied to PET/SPECT imaging with radiolabelled antibodies could similarly be adapted to generate well-defined chelator-antibody constructs. Several preclinical studies have highlighted that site-specifically modified antibodies have improved in vivo behaviour (higher affinity for target receptors, lower off target accumulation/persistence, better conjugate stability) relative to those modified using conventional, less specific methods. Site-specifically radiolabelled antibody-based radiopharmaceuticals will deliver new clinically-useful contrast agents for molecular PET/SPECT imaging, by (i) providing clinicians with better molecular imaging tools to predict whether a patient will respond to a particular treatment or intervention, and (ii) providing scientists with reliable tools to quantitatively map
the in vivo behaviour of new antibody-based therapies and/or newly-discovered receptors that are drug targets. Such technology will also be critically important in providing antibody conjugates with precisely defined structures and stoichiometries that are acceptable to regulatory authorities.

Acknowledgements

MM and MTM acknowledge the support of the Wellcome Trust (201959/Z/16/Z), a Cancer Research UK Career Establishment Award (C63178/A24959), the KCL and UCL Comprehensive Cancer Imaging Centre funded by CRUK and EPSRC in association with the MRC and DoH (England), and the NIHR Biomedical Research Centre at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the DoH.

References

[1] Vidarsson G, Dekkers G, Rispen T. IgG subclasses and allotypes: from structure to effector functions. Front Immunol 2014;5:520.
[2] Weiner GJ. Building better monoclonal antibody-based therapeutics. Nat Rev Cancer 2015;15:361.
[3] Mayes PA, Hance KW, Hoos A. The promise and challenges of immunogenic antibody development in cancer. Nat Rev Drug Discov 2018;17:509.
[4] Shen K, Ma X, Zhu C, Wu X, Jia H. Safety and efficacy of trastuzumab Emtansine in advanced human epidermal growth factor receptor 2-positive breast cancer: a meta-analysis. Sci Rep 2016;6:23262.
[5] Beck A, Goetsch L, Dumontet C, Corvaja N. Strategies and challenges for the next generation of antibody-drug conjugates. Nat Rev Drug Discov 2017;16:315-37.
[6] Younes A, Yasanuth U, Kirkpatrick P. Brentuximab vedotin. Nat Rev Drug Discov 2012;11:19.
[7] McDevitt MB, Thorek DJ, Hashimoto T, Gondo T, Veitch DR, Sharma SK, et al. Feed-forward alpha particle radiotherapy ablates androgen receptor-addicted prostate cancer. Nat Commun 2018;9:1629.
[8] Milenic DE, Brady ED, Brechbiel MW. Antibody-targeted radiation cancer therapy. Nat Rev Drug Discov 2004;4:488.
[9] Gebhart G, Lamberts BE, Wilmana Z, Garcia C, Emonts P, Ameye L, et al. Molecular imaging as a tool to investigate heterogeneity of advanced HER2-positive breast cancer and to predict patient outcome under trastuzumab emtansine (TDM1): the ZEPHIR trial. Ann Oncol 2016;27:619-24.
[10] Yasuda T, Palacios IF, Dec GW, Fallon JT, Gold HK, Leinbach RC, et al. Indium 111-monoclonal antymyosin antibody imaging in the diagnosis of acute myocarditis. Circulation 1987;76:306–11.
[11] Goldenberg DM, DeLand F, Kim E, Bennett S, Primus FJ, van Nagell JR, et al. Use of radiolabeled antibodies to carcoenymobryn antigens for the detection and localization of diverse cancers by external photoscanning. N Engl J Med 1979;289:1384–6.
[12] Yan R, Sander K, Galante E, Rajkumar V, Badar A, Robson M, et al. One-pot three-component radiochemical reaction for rapid assembly of [125I]-labeled molecular probes. J Am Chem Soc 2013;135:703–9.
[13] Adam MJ, Willbur DS. Radiolabeling for imaging and therapy. Chem Soc Rev 2005;34:153–63.
[14] Freise AC, Wu AM. In vivo imaging with antibodies and engineered fragments. Mol Immunol 2015;67:142–52.
[15] Schumacher D, Helma J, Schneider AFL, Leonhardt H, Hackenberger CPR. Nanobodies chemical functionalization strategies and intracellular applications. Angew Chem Int Ed 2018;57:2314–33.
[16] Ma MT, Blower PJ. Chelators for diagnostic molecular imaging with radioisotopes of copper, gallium and zirconium. In: Crichton R, Ward RJ, Hider RC, editors. Metal chelation in medicine. The Royal Society of Chemistry; 2017. p. 260-312.
[17] Ma MT, Donnelly PS. Peptide targeted copper-64 radiopharmaceuticals. Curr Top Med Chem 2011;11:500–20.
[18] Cusnier R, Imbert C, Hider R, Blower P, Ma M. Hydroxypropyridine chelators: from iron scavenging to radiopharmaceuticals for PET imaging with gallium-68. Int J Mol Sci 2017;18:116.
[19] Blower PJ. A nuclear chocolate box: the periodic table of nuclear medicine. Dalton Trans 2015;44:4819–44.
[20] Price EW, Orvig C. Matching chelators to radionuclides for radiopharmaceuticals. Chem Soc Rev 2014;43:260–90.
[21] Wadas T, Wong EH, Weisman GR, Anderson CJ. Coordinating radionuclides of copper, gallium, indium, yttrium, and zirconium for PET and SPECT imaging of disease. Chem Rev 2010;110:2858–902.
[22] Cohen R, Vugts DJ, Stigter-van Walsum M, Visser GWM, van Dongen G. Inert coupling of IRDye800CW and zirconium-89 to mononclonal antibodies for single- or dual-mode fluorescence and PET imaging. Nat Protoc 2013;8:1010–8.
[23] Vosjan M, Perk LR, Visser GWM, Budde M, Jurek P, Kiefer GE, et al. Conjugation and radio-labeling of mononclonal antibodies with zirconium-89 for PET imaging using the bifunctional chelate p-isothiocyanato benzyl-desferrioxime. Nat Protoc 2010;5:739–43.
[24] Zeglis BM, Lewis JS. A practical guide to the construction of radiometalled bioconjugates for positron emission tomography. Dalton Trans 2011;40:6168–95.
[25] Cooper MS, Ma MT, Sunasse K, Shaw KP, Williams JD, Paul RL, et al. Comparison of [111]Cu-complexing bifunctional chelators for radioimmunoconjugation: labeling efficiency, specific activity, and in vitro/in vivo stability. Bioconjug Chem 2012;23:1029–39.
[26] Ma MT, Meszaros LK, Paterson BM, Berry DJ, Cooper MS, Ma Y, et al. Tripodal tris(hydroxypropridinyl) ligands for immunoconjugate PET imaging with [18F]F: comparison with desferrioxime-B. Dalton Trans 2015;44:4884–900.
[27] Junutula JR, Raab H, Clark S, Bhaktas S, Leipold DD, Weir S, et al. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. Nat Biotechnol 2008;26:925.
[28] Boswell CA, Mundo EE, Zhang G, Bumbaca D, Valle NR, Kozak KR, et al. Impact of drug conjugation on pharmacokinetics and tissue distribution of anti-STEAP1 antibody-drug conjugates in rats. Bioconjug Chem 2011;22:1994–2004.
[29] Giersing BK, Rae MT, CarballidoBrea M, Williamson RA, Blower PJ. Synthesis and characterization of [111]In-DTPA-N-TIMP-2: a radiopharmaceutical for imaging matrix metalloproteinase expression. Bioconjug Chem 2001;12:964–71.
[30] Zeglis BM, Davis CB, Aggeler R, Kang HC, Chen AM, Agnew BJ, et al. Enzyme-mediated methodology for the site specific radiolabeling of antibodies based on catalyst-free click chemistry. Bioconjug Chem 2013;24:1057–67.
[31] Zhou Q, Stefano JE, Manning C, Kyazike J, Chen B, Gianollo DA, et al. Site-specific antibody-drug conjugation through glycoengineering. Bioconjug Chem 2014;25:510–20.
[32] Behrens CR, Liu B. Methods for site-specific drug conjugation to antibodies. Mabs 2014;6:46–53.
[33] Agarwal P, Bertozzi CR. Site-specific antibody-drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. Bioconjug Chem 2015;26:176–92.
[34] Hallam T, Wold E, Wahl A, Smider VV. Antibody conjugates with unnatural amino acids. Mol Pharm 2015;12:1848–62.
[35] Koniev O, Wagner A. Developments and recent advancements in the field of endogenous amino acid selective bond forming reactions for bioconjugation. Chem Soc Rev 2015;44:5495–551.
[36] Bouteurice O, Bernardez GJL. Advances in chemical protein modification. Chem Rev 2015;115:2174–95.
[37] Thompson P, Ezeadi E, Hutchinson I, Fleming R, Beazeb B, Lin J, et al. Straightforward glycoengineering approach to site-specific antibody-pyrolobenzodiazepine conjugates. ACS Med Chem Lett 2016;7:1005–8.
[38] Chudasama V, Maruani A, Cadick S. Recent advances in the construction of antibody-drug conjugates. Nat Chem 2016;8:114–9.
Simple, radioactive immunoconjugates for tumor uptake and imaging. Bioconjug Chem 2015;26:145−52.

Lyons RP, Setter J, Roves TD, Donorionna S, Hunter JH, Anderson ME, et al. Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates. Nat Biotechnol 2014;32:1059−62.

Smith BE, Caspersen MR, Robinson E, Maruani A, Nunes JM, et al. A platform for efficient, thiol-stable conjugation to albumin's native single accessible cysteine. Org Biomol Chem 2015;13:7946−9.

Toda N, Asano S, Barbas CF. Rapid, stable, chemoselective labeling of thiols with Julia-Kocienski-like reagents: a serum-stable alternative to maleimide-based protein conjugation. Angew Chem Int Ed 2013;52:12592−96.

Adameu P, Davydova M, Zeglis BM. Thiol-reactive bifunctional chelators for the creation of site-selectively modified radioimmunoconjugates with improved stability. Bioconjug Chem 2018;29:1364−72.

Nunes JPM, Vasileva V, Robinson E, Morais M, Smith MEB, Pedley RB, et al. Use of a next generation maleimide in combination with THIOMAB™ antibody technology delivers a highly stable, potent and near homogeneous THIOMAB™ antibody-drug conjugate (TDC). RSC Adv 2017;24828−32.

Bernardim B, Cal P, Matos MJ, Oliveira BL, Martinez-Saez N, Albuquerque IS, et al. Stoichiometric and irreversible cysteine-selective protein modification using carboxylic reactive agents. Nat Commun 2016;7.

Hamblett KJ, Senter PD, Chace DF, Sun MMC, Lenox J, Cerveny CG, et al. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. Clin Cancer Res 2004;10:7063−70.

Maruani A, Smith MEB, Miranda E, Chester KA, Chudasama V, Caddick S. A plug-and-play approach to antibody-based therapeutics via a chemoselective dual click strategy. Nat Commun 2015;6.

Wang T, Ng YZ, Liu JX, Xu XM, Xu YY, et al. Water-soluble allyl sulfoxones for dual site-specific labelling of proteins and cyclic peptides. Chem Sci 2016;7:3234−9.

Kuan SL, Wang T, Weil T. Site-selective disulfide modification of proteins: expanding diversity beyond the proteome. Chem Eur J 2016;22:17112−29.

Broccini S, Godwin A, Balan S, Choi JW, Zoh M, Shaunsak S. Disulfide bridge based PEylation of proteins. Adv Drug Deliv Rev 2008;60:3−12.

Shaunak S, Godwin A, Choi JW, Balan S, Pedone E, Vijayarangam D, et al. Site-specific PEylation of native disulfide bonds in therapeutic proteins. Nat Chem Biol 2006;2:312−3.

Wang T, Wu YZ, Kuan SL, Dumele O, Lamla M, Ng DYW, et al. A disulfide intercalator toolbox for the site-directed modification of polypeptides. Chem Eur J 2015;21:228−29.

Wang T, Ng DYW, Wu YZ, Thomas J, TamTran T, Weil T. Bis-sulfide bioconjugates for glutathione triggered tumor responsive drug release. Chem Commun 2014;50:1116−8.

Badescu G, Bryant P, Bird M, Henseleit K, Swierkosz J, Parekh V, et al. Bridging disulfides for stable and defined antibody drug conjugates. Bioconjug Chem 2014;25:1124−36.

Martinez-Saez N, Sun S, Oldrini D, Sormanni P, Bouteireaux O, Carboni F, et al. Oxetane grafts installed site-selectively on native disulfides to enhance protein stability and activity in vivo. Angew Chem Int Ed 2017;56:14963−67.
[82] Wilson P, Anastasakis A, Owen MR, Kempe K, Haddleton DM, Mann SK, et al. Organic arsenicals as efficient and highly specific linkers for protein/peptide-polymer conjugation. J Am Chem Soc 2015;137:4215–22.

[83] Rahou C, Richards DA, Maruani A, Love EA, Javaid F, Caddock S, et al. Highly homogeneous antibody modification through optimisation of the synthesis and conjugation of functionalised dibromopyridazinones. Org Biomol Chem 2018;16:1359–66.

[84] Bryden F, Maruani A, Savoie H, Chudasama V, Smith MEB, Caddock S, et al. Regioselective and stochiometrically controlled conjugation of photodynamic sensitizers to a HER2 targeting antibody fragment. Bioconjug Chem 2014;25:611–7.

[85] Nunes JPM, Morais M, Vassileva V, Robinson E, Rajkumar VS, Smith MEB, et al. Functional native disulfide bridging enables delivery of a potent, stable and targeted antibody-drug conjugate (ADC). Chem Commun 2015;51:10624–27.

[86] Schumacher FF, Nunes JPM, Maruani A, Chudasama V, Smith MEB, Chester KA, et al. Next generation maleimides enable the controlled assembly of antibody-drug conjugates via native disulfide bond bridging. Org Biomol Chem 2014;12:7261–9.

[87] Schumacher FF, Sanchanva VA, Tolner B, Wright ZVF, Ryan CP, Smith MEB, et al. Homogeneous antibody fragment conjugation by disulfide bridging introduces ‘spinocists’. Sci Rep 2013;3:1525.

[88] Smith MEB, Schumacher FF, Ryan CP, Tedaldi LM, Papaioannou D, Wakman G, et al. Protein modification, bioconjugation, and disulfide bridging using bromomaleimides. J Am Chem Soc 2010;132:1960–5.

[89] Morais M, Nunes JPM, Karu K, Forte F, Benni I, Smith MEB, et al. Optimisation of the dibromomaleimide (DBM) platform for native antibody conjugation by accelerated post-conjugation hydrolysis. Org Biomol Chem 2017;2947–52.

[90] Castañeda I, Maruani A, Schumacher FF, Miranda E, Chudasama V, Chester KA, et al. Acid-cleavable thiomaleic acid linker for homogenous antibody-drug conjugation. Chem Commun 2013;49:8187–9.

[91] Maguire RT, Pascucci VL, Maroli AN, Gulfo JV. Immunochemistry in patients with colorectal, ovarian, and prostate cancer. Results with site-specific immunocloners. Cancer 1993;72:3453–62.

[92] Bohdiewicz PJ. Indium-111 satumomab pimektide: the first FDA-approved monoclonal antibody for tumor imaging. J Nucl Med Technol 1998;26:155–63.

[93] Taneja SS. ProstaScint® scan: contemporary use in clinical practice. Rev Urol 2004;6:519–28.

[94] Young JD, Abbate V, Imberti C, Meszaros LK, Ma MT, Terry SYA, et al. 60Ga-Tg-PSMA-a PET imaging agent for prostate cancer offering rapid, room-temperature, one-step kit-based radiolabelling. J Nucl Med 2017;58:1270–7.

[95] Afshar-Oromieh A, Malcher A, Eder M, Eisenhut M, Linhart HG, Hadaschik BA, et al. PET imaging with a 111In-labelled PSMA ligand for the diagnosis of prostate cancer: biodistribution in humans and first evaluation of tumour lesions. Eur J Nucl Med Mol Imaging 2013;40:486–95.

[96] Robu S, Schottelius M, Eiber M, Maurer T, Gschwend J, Schwaiger M, et al. Preclinical evaluation and first patient application of 99mTc-PSMA-I65 for SPECT imaging and radioguided surgery in prostate cancer. J Nucl Med 2017;58:235–42.

[97] Hofman MS, Eu P, Jackson P, Hong E, Binns D, Iravani A, et al. Cold kit for prostate-specific membrane antigen (PSMA) PET imaging: phase 1 study of 68Ga-tris(hydroxypyridinone)-PSMA PET/CT in patients with prostate cancer. J Nucl Med 2018;59:625–31.

[98] Oshannessy DJ, Doberen MJ, Quares RL. A novel procedure for labeling immunoglobulins by conjugation to oligosaccharide moieties. Immunol Lett 1984;8:273–7.

[99] Schwarz U, Wunderlich G, Brossmer R. Transfer of 111In and fluoresceinyl sulfonic acid derivatives into the oligosaccharide chains of IgG: a new method for site-specific labeling of antibodies. Nucl Med Biol 1999;26:383–8.

[100] Hu MD, Chen P, Wang J, Chan C, Scollard DA, Reilly RM. Site-specific conjugation of HIV-1 tat peptides to IgG: a potential route to construct radioimmunoconjugates for targeting intracellular and nuclear epitopes in cancer. Eur J Nucl Med Mol Imaging 2006;33:301–10.

[101] Rodwell JD, Alvarez VL, Lee C, Lopes AD, Goers JW, King HD, et al. Site-specific covalent modification of monoclonal antibodies: in vitro and in vivo evaluations. Proc Nat Acad Sci U S A 1986;83:2632–6.

[102] Rosenstrauss MJ, Davis WL, Lopes AD, Daleo CJ, Gilman SC. Immunohistochemical and pharmacokinetic characterization of site-specific immunomonoconjugates 15AS-glycyl-tyrosyl-(N-epsilon-dithylenetriamine pentaacetic acid) -lysine derived from anti-breast carcinoma monoclonal antibody 15AS. Cancer Res 1991;51:5744–51.

[103] Chengazi VU, Feneley MR, Ellison D, Stalsett M, Gronowski A, Granowska M, et al. Imaging prostate cancer with technetium-99m-7E11.C5.3 (CYT-351). J Nucl Med 1997;38:675–82.

[104] Stalteri MA, Mather SJ, Belinka BA, Coughlin DJ, Chengazi VU, Britton KE. Site-specific conjugation and labelling of prostate antibody 7E11.C5.3 (CYT-351) with technetium-99m. Eur J Nucl Med 1997;24:651–4.

[105] Jeong JM, Lee J, Paik CH, Kim DK, Lee DS, Chung JK, et al. Site-specific 99mTc-labeling of antibody using dihydroazirinophthalazine (DHZ) conjugation to Fe region of heavy chain. Arch Pharm Res 2004;27:961–7.

[106] Jolley C, Burnet FR, Blow PR. Improved synthesis and characterisation of a hydrazide derivative of diethylentriaminepentaacetic acid for site-specific labelling of monoclonal antibodies with 111In. Appl Radiat Isot 1996;47:623–6.

[107] Kalia J, Raines RT. Hydrolytic stability of hydrazones and oximes. Angew Chem Int Ed 2008;47:7523–6.

[108] Bejot R, Goggi J, Moonshin SS, Padmanabhan P, Bhakoo KK. Aminoxy-functionalized DOTA for radioiodination of antibodies: evaluation of site-specific 111In-labeled trastuzumab. J Label Compd Radiopharm 2012;55:346–53.

[109] Wang WR, Vlasak J, Li YS, Pristatys P, Fang YL, Pittman T, et al. Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies. Mol Immunol 2011;48:860–6.

[110] Zeglis BM, Davis CB, Abdel-Attii D, Carlin SD, Chen AM, Aggerle R, et al. Chemoenzymatic strategy for the synthesis of site-specifically labeled immunomonoconjugates for multimodal PET and optical imaging. Bioconjug Chem 2014;25:2123–8.

[111] Fontana A, Spolaore B, Mero A, Veronese FM. Site-specific modification and PEGylation of pharmaceutical proteins mediated by transglutaminase. Adv Drug Deliv Rev 2008;60:13–28.

[112] Wu P, Shui W, Carlson BL, Hu N, Rabuka D, Lee J, et al. Site-specific chemical modification of recombinant proteins produced in mammalian cells by using the genetically encoded aldehydyl tag. Proc Nat Acad Sci U S A 2009;106:3000.

[113] Popp MW-L, Poepl HL. Making and breaking peptide bonds: protein engineering using sortase. Angew Chem Int Ed 2011;50:5024–32.

[114] Peters W, Willnow S, Duiskens M, Kleine H, Macherey T, Duncan KE, et al. Enzymatic site-specific functionalization of protein methyltransferase substrates with alkynes for click labeling. Angew Chem Int Ed 2010;49:5170–3.

[115] Jeger S, Zimmermann K, Blanc A, Grünberg J, Honer M, Hunziker P, et al. Site-specific and stoichiometric modification of antibodies by bacterial transglutaminase. Angew Chem Int Ed 2010;49:9995–7.

[116] Grünberg J, Jeger S, Sarko D, Dennler P, Zimmermann K, Mier W, et al. DOTA-Functionalized polylysine: a high number of DOTA chelates positively influences the biodistribution of enzymatic conjugated anti-tumor antibody chCL7a4g. PLoS One 2013;8.

[117] Chen L, Cohen J, Song X, Zhao A, Ye Z, Feulner CJ, et al. Improved variants of SRTa for site-specific conjugation on antibodies and proteins with high efficiency. Sci Rep 2016;6:31899.

[118] Alt K, Paterson BM, Westein E, Rudd SE, Poniger SS, Jagdale S, et al. A versatile approach for the site-specific modification of recombinant antibodies using a combination of enzyme-mediated bioconjugation and click chemistry. Angew Chem Int Ed 2015;54:7513–9.

[119] Paterson BM, Alt K, Jeffery CM, Price RL, Jagdale S, Rigby S, et al. Enzyme-mediated site-specific bioconjugation of metal complexes to proteins: sortase-mediated coupling of copper-64 to a single-chain antibody. Angew Chem Int Ed 2014;53:6115–9.
[120] Vaneycken I, Devoogdt N, Van Gassen N, Vincke C, Xavier C, Wernery U, et al. Preclinical screening of anti-HER2 nanobodies for molecular imaging of breast cancer. FASEB J 2011;25:2433–46.

[121] Kogelberg H, Miranda E, Burnet J, Ellison D, Tolner B, Foster J, et al. Generation and characterization of a diabody targeting the αvβ₆ integrin. PLOS One 2013;8.

[122] Kampmeier F, Williams JD, Maher J, Mullen GE, Blower PJ. Design and preclinical evaluation of a [⁹⁹ᵐTc]-labelled diabody of mAb JS91 for SPECT imaging of prostate-specific membrane antigen (PSMA). EJNMRI Res 2014;4.

[123] Xavier C, Devoogdt N, Hernot S, Vaneycken I, D’Huyvetter M, De Vos J, et al. Site-specific labeling of His-tagged Nanobodies with [⁹⁹ᵐTc]: a practical guide. Methods Mol Biol 2012;911:485–90.

[124] Waibel R, Alberto R, Willuda J, Finnern R, Schibili R, Stichelberger A, et al. Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel Tc(III)-carbonyl complex. Nat Biotechnol 1999;17:897.

[125] Willuda J, Honegger A, Waibel R, Schubiger PA, Stahel R, Zangemeister-Wittke U, et al. High thermal stability is essential for tumor targeting of antibody fragments. Cancer Res 1999;59:5758.

[126] Badar A, Williams J, de Rosales RTM, Tavaré R, Kampmeier F, Blower PJ, et al. Optimising the radiolabelling properties of technetium tricarbonyl and His-tagged proteins. EJNMRI Res 2014;4:14.

[127] Schibili R, Schubiger AP. Current use and future potential of organometallic radiopharmaceuticals. Eur J Nucl Med Mol Imaging 2002;29:1529–42.

[128] Tavaré R, Williams J, Howland K, Blower PJ, Mullen GED. [Ret(CO)₃]⁺ labelling of a novel cysteine/hexahistidyl tag: insights into binding mode by liquid chromatography-mass spectrometry. J Inorg Biochem 2012;114:24–7.

[129] Höfström C, Alital M, Honarvar H, Strand J, Malmberg J, Hosseinimehr SJ, et al. Hahana, Hehehi, Hiihihi, or HKHHKH. Influence of position and composition of histidine containing tags on biodistribution of [⁹⁹ᵐTc(CO)₃]⁺-labeled affibody molecules. J Med Chem 2013;56:4966–74.

[130] Francesconi LC, Zheng Y, Bartis J, Blumenstein M, Costello C, De Rosch MA. Preparation and characterization of [⁹⁹ᵐTcO]⁺ aptide: a technetium labeled peptide. Inorg Chem 2004;43:2867–75.

[131] Chen J, Cheng Z, Hoffman TJ, Jurisson SS, Quinn TP. Melanoma-targeting properties of [⁹⁹ᵐTc]Technetium-labeled cyclic α-melanocyte-stimulating hormone peptide analogues. Cancer Res 2000;60:5649.

[132] George AJT, Jamar F, Tai MS, Heelan BT, Adams GP, McCartney JE, et al. Radiometal labeling of recombinant proteins by a genetically engineered minimal chelation site: technetium-99m coordination by single-chain Fv antibody fusion proteins through a C-terminal cysteiny1 peptide. Proc Nat Acad Sci U S A 1995;92:8358–62.

[133] Tran T, Engfeldt T, Orlova A, Widström C, Bruskin A, Tolmachev V, et al. In vivo evaluation of cysteine-based chelators for attachment of [⁹⁹ᵐTc] to tumor-targeting affibody molecules. Bioconjug Chem 2007;18:549–58.

[134] Ahlgren S, Wällberg H, Tran TA, Widström C, Hjertman M, Abrahamsen L, et al. Targeting of HER2-expressing tumors with a site-specifically [⁹⁹ᵐTc]-labeled recombinant affibody molecule, ZHER2:2395, with C-terminally engineered cysteine. J Nucl Med 2009;50:781–9.

[135] Ahlgren S, Andersson K, Tolmachev V. Kit formulation for [⁹⁹ᵐTc]-labeling of recombinant anti-HER2 Affibody molecules with a C-terminally engineered cysteine. Nucl Med Biol 2010;37:539–46.

[136] Berndorff D, Borkowski S, Moosmayer D, Viti F, Müller-Tiemand B, Sieger S, et al. Imaging of tumor angiogenesis using [⁹⁹ᵐTc]-labeled human recombinant anti-ED-B fibronectin antibody fragments. J Nucl Med 2006;47:1707–16.

[137] Levashova Z, Backer M, Backer JM, Blankenberg FG. Direct site-specific labeling of the Cys-tag moiety in scVEGF with technetium 99m. Bioconjug Chem 2008;19:1049–54.