

Enzyme-Mediated Site-Specific Bioconjugation of Metal Complexes to Proteins: Sortase-Mediated Coupling of Copper-64 to a Single-Chain Antibody**

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Dedicated to Professor Jack M. Harrowfield on the occasion of his 70th birthday

Abstract: The enzyme-mediated site-specific bioconjugation of a radioactive metal complex to a single-chain antibody using the transpeptidase sortase A is reported. Cage amine sarcophagine ligands that were designed to function as substrates for the sortase A mediated bioconjugation to antibodies were synthesized and enzymatically conjugated to a single-chain variable fragment. The antibody fragment scFv_{anti-LIBS} targets ligand-induced binding sites (LIBS) on the glycoprotein receptor GPIIb/IIIa, which is present on activated platelets. The immunoconjugates were radiolabeled with the positron-emitting isotope ⁶⁴Cu. The new radiolabeled conjugates were shown to bind selectively to activated platelets. The diagnostic potential of the most promising conjugate was demonstrated in an in vivo model of carotid artery thrombosis using positron emission tomography. This approach gives homogeneous products through site-specific enzyme-mediated conjugation and should be broadly applicable to other metal complexes and proteins.

The incorporation of selected radioactive isotopes into antibodies or antibody fragments combines the diagnostic and therapeutic possibilities of nuclear medicine with the exquisite selectivity of antibody targeting.^[1] Engineered antibody derivatives, such as Fab fragments and single-chain variable fragments (scFv), aim to maintain specificity and

selectivity with faster clearance from non-target tissue. A promising strategy to enable the use of metal radionuclides in antibody-targeted imaging and therapy is to design molecules that coordinate to the metal ion and preclude its release in vivo.^[1a,2] The attachment of the chelator to the targeted antibody or fragment requires careful consideration to avoid compromising the affinity of the antigen-binding site for the target. Chelating ligands have traditionally been attached to antibodies through non-specific chemical conjugation reactions with the ε-amine of lysine residues or the thiol groups of cysteine residues. The multiple lysine and cysteine residues of monoclonal and scFv antibodies result in the formation of heterogeneous products with various numbers of chelators attached. Conjugations involving residues that are critical for antigen binding can compromise biological activity and are particularly problematic for antibody fragments because of their smaller size. Herein, we utilize an enzymatic site-specific conjugation strategy to attach metal complexes to an scFv antibody. The techniques and concepts presented are broadly applicable to a wide range of metal-ion chelators, antibodies, and antibody fragments.

Imaging technologies for the visualization of cellular and molecular processes in patients (molecular imaging) can assist clinicians in both diagnosis and monitoring treatment. Among the molecular imaging modalities, positron emission tomog-

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B)

The transpeptidase sortase A (SrtA) is produced by Gram-positive bacteria and covalently attaches cell surface proteins to the cell wall.^[7] In bacteria, SrtA cleaves an amide bond between Thr and Gly in the small pentapeptide recognition motif Leu-Pro-*X*-Thr-Gly (where *X*=any amino acid).^[8] The Leu-Pro-*X*-Thr-Gly “sorting tag” can be included in recombinantly expressed proteins or antibodies to

Herein, we describe the synthesis of new sarcophagine derivatives designed to function as substrates for SrtA and demonstrate their conjugation to scFv_{anti-LIBS}, which is catalyzed by SrtA. One of the first challenges was that affinity chromatography with the use of a scFv_{anti-LIBS} cloned with a His6 affinity tag after the LPETGG motif (which is conveniently cleaved during the enzymatic conjugation)^[9a,c] was not possible as the sarcophagine chelator strips the Ni²⁺ from the purification column. This problem was circumvented by the use of a variant with a FLAG

affinity tag at the C terminus of scFv_{anti-LIBS} and the SrtA enzyme.^[14]

To enable site-specific conjugation to scFv_{anti-LIBS}-LPETGG-FLAG using SrtA, the bifunctional chelator MeCOSar^[15] (Figure 1A) was elaborated with glycine-containing short-chain oligo(ethylene glycol) (OEG) linkers.^[16] The scope and tolerance of the SrtA mediated enzymatic reaction was investigated with the chelator MeCOSar with four different peptide functional groups. The first step in the synthesis of **L**¹-**L**³ was the protection of a single amine of 4,7,10-trioxatridecane-1,13-diamine by reaction with ethyl-trifluoroacetate. In the next step, this amine was added to the *N*-hydroxysuccinimide activated (*t*Boc)₄₋₅MeCOSar-NHS ester, which was followed by the removal of the trifluoroacetamide group. (Figure 1A). The bifunctional chelators **L**¹ (no Gly residue), **L**² (one Gly residue), and **L**³ (three Gly residues) were characterized by NMR spectroscopy and ESI mass spectrometry. The ¹³C NMR spectra, which were acquired in D₂O, indicated the presence of the glycine CH₂ groups for both **L**² (δ = 41.0 ppm) and **L**³ (δ = 41.1, 43.0, and 43.2 ppm). Aside from the aminoglycine short-chain OEG linkers, a triglycine-containing peptide (GGGWSSK), which has been shown to be an efficient SrtA substrate,^[9a] was also attached to MECOSar through the ϵ -amino group of the C-terminal lysine residue using solid-phase peptide synthesis and 9-fluorenylmethyloxycarbonyl (Fmoc) and (4-methoxyphenyl)(diphenyl)methyl (Mmt) protecting groups to give **L**⁴ (Figure 1B).

Each bifunctional chelator was allowed to react with scFv_{anti-LIBS}-LPETGG-FLAG in the presence of SrtA for five hours at 37 °C (Figure 1C). The products were purified by anti-FLAG affinity chromatography, and their identity and purity were confirmed with SDS-PAGE page and ESI mass spectrometry (SDS-PAGE = sodium dodecylsulfate-polyacrylamide gel electrophoresis; Figure 2). The reaction with **L**¹ resulted in a poor yield (ca. 30 %). The best yields were obtained using **L**² and **L**⁴ (ca. 90 %). The construct from **L**³ was obtained in poor yield (ca. 50 %) despite the presence of three Gly residues.

The sortase-mediated coupling is reversible because the products **L**²-**L**⁴ contain LPETG sequences. It is possible that the good yield of scFv_{anti-LIBS}-LPET-**L**² is due to the presence of the OEG linker directly next to the single glycine residue, which impairs the reverse reaction. The product scFv_{anti-LIBS}-LPET-**L**⁴ was generated in good yield because of the presence of Trp residues (LPETGGGW), which slows the rate of the reverse reaction, presumably because of the steric bulk of the indole side chain.^[17] The deconvoluted ESI mass spectra of scFv_{anti-LIBS}-LPET-**L**² and scFv_{anti-LIBS}-LPET-**L**⁴ are shown in Figure 2, with the mass-to-charge (*m/z*) distributions centered at 31 064 Da and 31 650 Da, respectively (**L**²: 687 g mol⁻¹; **L**⁴: 1273 g mol⁻¹). The selectivity of the immunoconjugates for platelets that were activated by the addition of adenosine diphosphate (20 μ M) was assessed using fluorescence-activated cell sorting (FACS), which indicated strong binding to the target (Figure 2; see also the Supporting Information). None of the constructs bound to non-activated platelets. Both **L**² and **L**⁴ gave good yields in SrtA mediated reactions; however, as **L**² is easier to synthesize and has

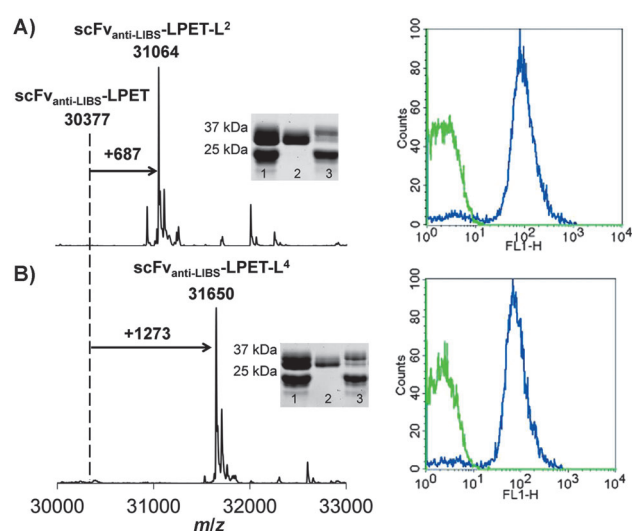


Figure 2. A, B) ESI mass spectra of scFv_{anti-LIBS}-LPET-**L**² (A) and scFv_{anti-LIBS}-LPET-**L**⁴ (B). Insets: SDS-PAGE analysis of 1) the non-purified reaction mixture; 2) purified scFv_{anti-LIBS}-LPET-**L**² (A) and scFv_{anti-LIBS}-LPET-**L**⁴ (B); and 3) SrtA (*M*_w = 17.9 kDa) and scFv_{anti-LIBS}-LPETGG-FLAG (*M*_w = 33.5 kDa) after elution from the anti-FLAG affinity chromatography column. Right-hand side: FACS histograms showing the binding of scFv_{anti-LIBS}-LPET-**L**² (A) and scFv_{anti-LIBS}-LPET-**L**⁴ (B) to activated platelets (blue line) and non-activated platelets (green line).

a lower molecular weight, it was used for further studies. Aside from scFv_{anti-LIBS}-LPET-**L**², a mutant variant (scFv_{mut}-LPET-**L**²) was synthesized using SrtA mediated conjugation and used as a control for determining the degree of antigen specificity. The mutant variant is based on an Arg-Gly-Asp (RGD) mutation of the parent antibody resulting in complete loss of binding to GPIIb/IIIa.^[18]

The immunoconjugates were radiolabeled with ⁶⁴Cu²⁺ at pH 7 and room temperature in less than 30 minutes with a radiochemical purity of > 95 %. The antigen binding of the conjugate with ⁶⁴Cu²⁺ coordinated to the chelator was comparable to that of copper-free scFv_{anti-LIBS}-LPET-**L**² and the unmodified scFv_{anti-LIBS}, and neither construct bound to non-activated platelets (data not shown).

The targeting abilities of the immunoconjugates scFv_{anti-LIBS}-LPET-⁶⁴Cu**L**² and scFv_{mut}-LPET-⁶⁴Cu**L**², which were radiolabeled with ⁶⁴Cu, towards activated platelets were evaluated in an in vivo model of mouse carotid artery thrombosis, which was induced by exposure to FeCl₃.^[9c, 10a,c, 13, 19] The biodistribution data confirmed a significant increase in scFv_{anti-LIBS}-LPET-⁶⁴Cu**L**² uptake in the injured vessel (34.01 ± 7.9 % ID/g) compared to the non-injured vessel (14.25 ± 5.5 % ID/g, *p* = 0.0001) and the muscle as control (0.59 ± 0.36 % ID/g, *p* = 0.0001; Figure 3). In the injured vessel, the uptake of scFv_{anti-LIBS}-LPET-⁶⁴Cu**L**² was significantly higher than that of scFv_{mut}-LPET-⁶⁴Cu**L**² (13.03 ± 11.48 % ID/g, *p* = 0.0001). In a separate study, administration of scFv_{anti-LIBS} (400 μ g) 30 minutes before radiotracer injection to block the binding sites significantly reduced the binding of scFv_{anti-LIBS}-LPET-⁶⁴Cu**L**² to the target receptor (*p* = 0.0001; Figure 3). The mice were placed in a small-animal PET/CT scanner (CT = computed tomogra-

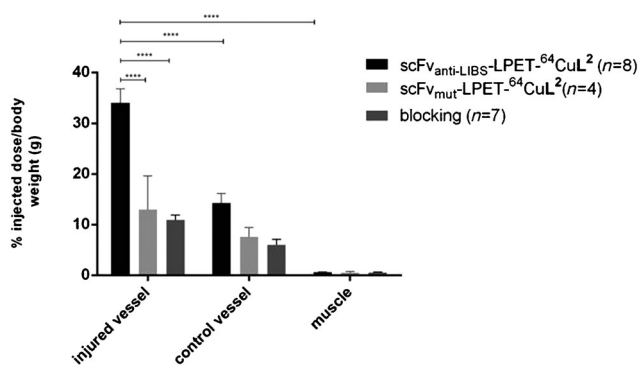


Figure 3. Biodistribution of scFv_{anti-LIBS}-LPET-⁶⁴CuL² (black) and scFv_{mut}-LPET-⁶⁴CuL² (light grey) in the injured vessel compared to the non-injured vessel and the muscle. Blocking of scFv_{anti-LIBS}-LPET-⁶⁴CuL² uptake (dark grey) in an in vivo model of mouse carotid artery thrombosis with 400 µg of non-radioactive scFv_{anti-LIBS}, which was administered 30 minutes before radiotracer injection. Data are given as the percentage of the injected dose per body weight (g) and were measured 90 minutes after tracer injection (****/*p* = 0.0001).

phy) 30 minutes after injection of a single dose of scFv_{anti-LIBS}-LPET-⁶⁴CuL² or scFv_{mut}-LPET-⁶⁴CuL² (3–5 MBq) with the carotid artery set as the region of interest. The PET/CT scans showed an accumulation of scFv_{anti-LIBS}-LPET-⁶⁴CuL² in the injured vessel and no significant uptake of scFv_{mut}-LPET-⁶⁴CuL², which confirms that GPIIb/IIIa mediated binding is highly specific (Figure 4).

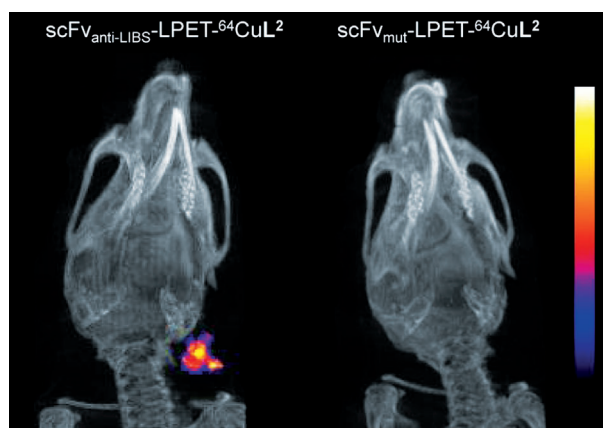


Figure 4. Serial small-animal PET/CT images of an in vivo model of mouse carotid artery thrombosis. Comparison of representative maximum-intensity projection PET images of scFv_{anti-LIBS}-LPET-⁶⁴CuL² and scFv_{mut}-LPET-⁶⁴CuL² 60 minutes after injection of the radiotracer. The color scale for all PET image data shows radiotracer uptake with white and blue corresponding to the highest and lowest activity, respectively.

In summary, we have reported the synthesis and isolation of bifunctional chelators and demonstrated their ability for high-yielding, site-specific, and reproducible modification of an scFv antibody against the platelet integrin GPIIb/IIIa in its active ligand-bound form using SrtA. Sortase-mediated bioconjugation is particularly beneficial for modifying antibody fragments that lack the readily accessible and modifi-

able oligosaccharide side chains of full antibodies. The high level of specific binding to activated platelets was maintained upon enzyme-mediated conjugation and radiolabelling of the immunoconjugate products and suggests that they have the potential to be of use for the in vivo detection of activated platelets for early diagnosis of acute thrombotic events, such as myocardial infarction and ischemic stroke, as well as inflammation. Advances in antibody development have resulted in rapid growth in this area of human therapeutics and a dramatically increasing number of FDA-approved agents. Much of the experience gained from the generation and optimization of one antibody is applicable to other antibodies. The specificity and selectivity of antibodies can be exploited for PET imaging and targeted radiotherapy but the construction of radionuclide-antibody agents requires careful consideration to maximize biological efficacy. The approach described here gives rise to homogeneous products through site-specific and controlled enzyme-mediated conjugation and should be broadly applicable to other metal complexes and proteins.

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