

New Bifunctional Chelators Incorporating Dibromomaleimide Groups for Radiolabeling of Antibodies with Positron Emission Tomography Imaging Radioisotopes

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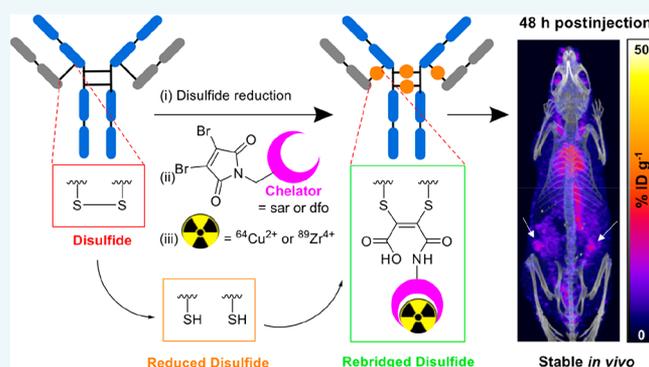
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ABSTRACT: Positron Emission Tomography (PET) imaging with antibody-based contrast agents frequently uses the radioisotopes $[^{64}\text{Cu}]\text{Cu}^{2+}$ and $[^{89}\text{Zr}]\text{Zr}^{4+}$. The macrobicyclic chelator commonly known as sarcophagine (sar) is ideal for labeling receptor-targeted biomolecules with $[^{64}\text{Cu}]\text{Cu}^{2+}$. The siderophore chelator, desferrioxamine-B (dfo), has been widely used to incorporate $[^{89}\text{Zr}]\text{Zr}^{4+}$ into antibodies. Here, we describe new bifunctional chelators of sar and dfo: these chelators have been functionalized with dibromomaleimides (dbm), that enable site-specific and highly stable attachment of molecular cargoes to reduced, solvent-accessible, interstrand native disulfide groups. The new sar–dbm and dfo–dbm derivatives can be easily conjugated with the IgG antibody trastuzumab via reaction with reduced interstrand disulfide groups to give site-specifically modified dithiomaleamic acid (dtm) conjugates, sar–dtm–trastuzumab and dfo–dtm–trastuzumab, in which interstrand disulfides are rebridged covalently with a small molecule linker. Both sar– and dfo–dtm–trastuzumab conjugates have been radiolabeled with $[^{64}\text{Cu}]\text{Cu}^{2+}$ and $[^{89}\text{Zr}]\text{Zr}^{4+}$, respectively, in near quantitative radiochemical yield (>99%). Serum stability studies, *in vivo* PET imaging, and biodistribution analyses using these radiolabeled immunoconjugates demonstrate that both $[^{64}\text{Cu}]\text{Cu}$ –sar–dtm–trastuzumab and $[^{89}\text{Zr}]\text{Zr}$ –dfo–dtm–trastuzumab possess high stability in biological milieu. Dibromomaleimide technology can be easily applied to enable stable, site-specific attachment of radiolabeled chelators, such as sar and dfo, to native interstrand disulfide regions of antibodies, enabling tracking of antibodies with PET imaging.



Monoclonal IgG antibody therapies have been transformative in the treatment of many cancers and autoimmune diseases, and new antibody therapies are likely to have further clinical impact. The ability to quantitatively image antibody biodistribution at the whole-body level can help predict individual patient response, as well as aid in understanding treatment outcomes during clinical development.^{1,2} Positron-emitting radiometallic isotopes attached to antibodies via chelators have been widely used to image antibody biodistribution using Positron Emission Tomography (PET).

Conventional labeling strategies have attached chelators to antibodies using electrophilic groups such as isothiocyanates, *N*-hydroxysuccinimides, and anhydrides that react with solvent accessible primary amines of lysine side chains.^{3–5} These bioconjugation methods are simple, but as these reactions are not site-specific and lack stoichiometric control, the presence of multiple lysine side chains leads to heterogeneous product mixtures. The resulting chelator–antibody conjugates often demonstrate reduced affinity for target receptors, particularly at

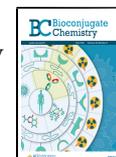
high chelator:antibody conjugation ratios.^{6–8} Several recent and elegant site-specific strategies include enzyme-mediated conjugation of chelators to (i) glycan regions of IgG antibodies using a combination of β -1,4-galactosidase/ β -1,4-galactosyltransferase(Y289L),^{9–11} (ii) hinge region glutamine residues of IgG antibodies using a combination of deglycosylation with *N*-glycosidase followed by chelator coupling with bacterial transglutaminase,¹² and (iii) modified C-termini of antibody fragment derivatives using the bacterial enzyme sortase A.¹³ Alternatively, incorporation of a non-natural amino acid containing an azide group in an IgG antibody has enabled orthogonal and site-selective conjugation

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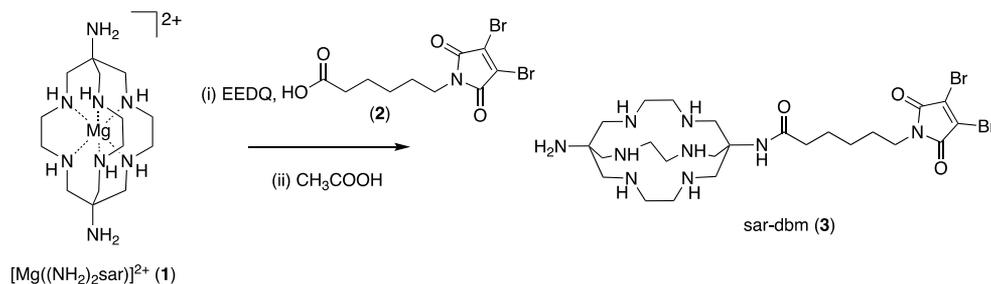
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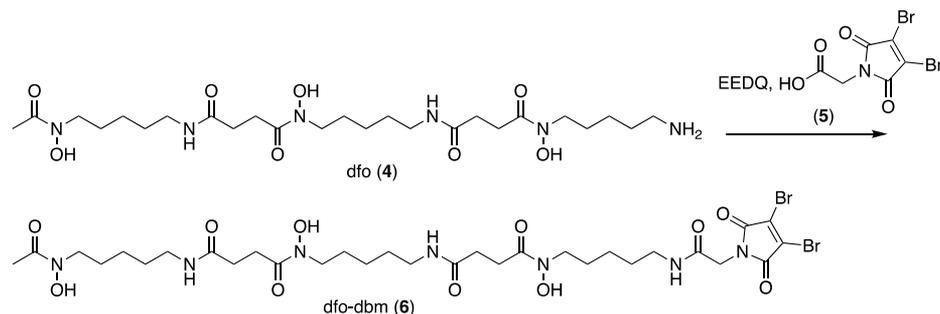
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Scheme 1. Synthesis of sar-dbm



Scheme 2. Synthesis of dfo-dbm



of chelators using click chemistry.¹⁴ These approaches have resulted in radiolabeled chelator–protein conjugates that display improved PET imaging capabilities relative to radiolabeled conjugates prepared using conventional, non-site-specific methods. However, these enzyme-based or protein engineering-based conjugation methods are more difficult to implement than conventional, non-site-specific conjugation technologies. Furthermore, modification or removal of glycan regions can modify the pharmacokinetics of antibodies, as well as affect their Fc-receptor binding properties and antibody-dependent cell-mediated toxicity.^{15–17}

Maleimide derivatives have been widely used to incorporate radiolabeled chelators into IgG antibodies and other proteins.^{18–21} Maleimides react selectively with reduced disulfides at near-neutral pH, and in the case of IgG antibodies, reduction of the four solvent-accessible, interstrand disulfide bonds leads to the presence of eight available thiol attachment points. While maleimides enable site-selective attachment of chelators and other cargoes to IgG antibodies, these conjugates are unstable: the resulting thioether can undergo a retro-Michael reaction in biological milieu, converting back to the starting thiol and maleimide.^{21–24} *In vivo*, the starting maleimide motif, still tethered to its payload, can then react with endogenous molecules containing bioavailable thiols, such as glutathione and albumin. In radionuclide imaging, this can potentially result in accumulation of radioactivity at off-target sites, decreasing image contrast, sensitivity, and the ability to quantify antibody distribution.

New conjugation platforms including bissulfone,²⁵ divinylpyrimidine,²⁶ divinyltriazine,²⁷ dibromoalkyl oxetane,²⁸ dibromopyridazinedione,^{29,30} and disubstituted maleimide^{31–36} derivatives enable site-specific attachment of cargo to dithiol groups of antibodies, including those at IgG hinge regions. Here, we focus on dibromomaleimide: this motif reacts specifically with two reduced thiol groups of antibodies, thus enabling concomitant attachment of cargo *and* rebridging of two cysteines. The resulting dithiomaleimide can be hydro-

lyzed to a dithiomaleamic acid under mildly basic conditions to give homogeneous antibody conjugates.^{32–35} Importantly, the dithiomaleamic acid conjugates are unreactive toward serum thiols and do not undergo retro-Michael reactions in biological media, unlike conventional maleimide derivatives. This dibromomaleimide platform has the potential to enable highly stable site-specific radiolabeling of antibodies at hinge region disulfides.

Zirconium-89 ($t_{1/2} = 78$ h, β^+ $E_{\max} = 897$ keV, 23%) and copper-64 ($t_{1/2} = 12.7$ h, β^+ $E_{\max} = 656$ keV, 18%) have both been used for imaging antibody distribution with PET: the half-lives of these isotopes match the time required for antibodies to clear circulation and accumulate in target tissue (1 day–1 week). Derivatives of a naturally occurring siderophore, desferrioxamine-B (dfo), are commonly used to incorporate [⁸⁹Zr]Zr⁴⁺ into monoclonal IgG antibodies for clinical and preclinical PET imaging.^{1,2,4,11,18,19} The dfo chelator contains three hydroxamate groups, enabling hexadentate O₆ coordination of Zr⁴⁺. Many chelators have been developed for [⁶⁴Cu]Cu²⁺ over the past three decades. Peptide and protein conjugates of the hexaazamacrocyclic chelator, 3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane, known commonly as “sarcophagine” (sar), have proved to be simple to radiolabel and are highly stable to demetalation *in vivo*, leading to high quality PET images.^{3,10,13,37–39} Here we describe preparation of dibromomaleimide derivatives of dfo and sar chelators and show the usability and applicability of this dibromomaleimide platform for site-specific radiolabeling of an IgG1 antibody with [⁸⁹Zr]Zr⁴⁺ and [⁶⁴Cu]Cu²⁺.

■ PREPARATION OF SAR-DBM AND DFO-DBM BIFUNCTIONAL CHELATORS

The 1,8-diamino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane, or “(NH₂)₂sar”, chelator is a useful precursor for preparation of bioconjugates. It contains two primary amines and six secondary amines, all of which are reactive toward electrophiles.⁴⁰ Prior studies have achieved selective functionalization

Scheme 3. Preparation of Chelator–dtm–Trastuzumab Immunoconjugates

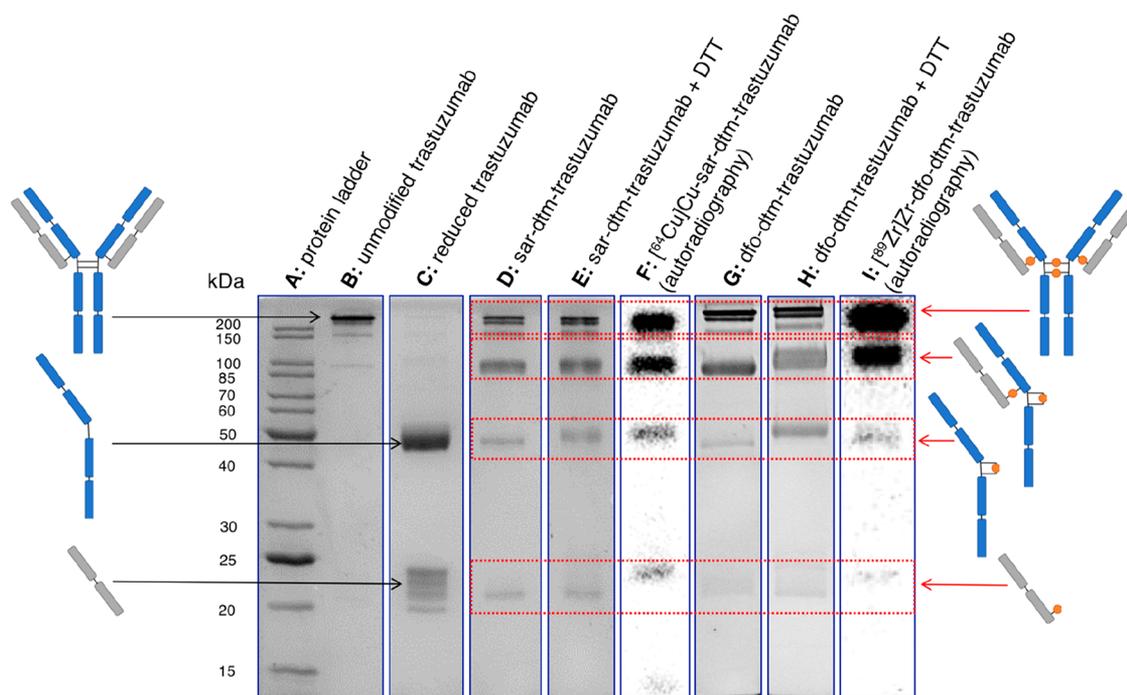
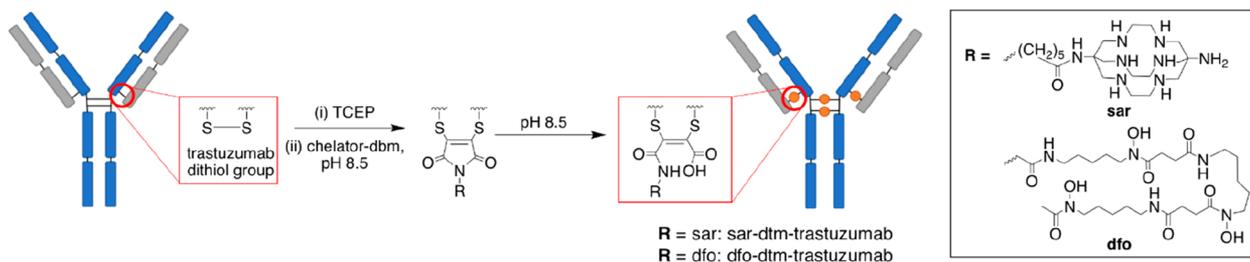


Figure 1. SDS-PAGE of chelator–dtm–trastuzumab conjugates. The full gels are included in Figures S3–S5.

of primary amines by “protecting” secondary amine groups through metal ion (Cu^{2+} or Mg^{2+}) complexation.^{37–39} We elected to use $[\text{Mg}((\text{NH}_2)_2\text{sar})]^{2+}$ (1) as a precursor,³⁹ as following derivatization, Mg^{2+} can be easily removed from the chelator by acidification. $[\text{Mg}((\text{NH}_2)_2\text{sar})]^{2+}$ was reacted with 3,4-dibromomaleimide-*N*-hexanoic acid (2) and coupling agent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in acetonitrile (Scheme 1). The resulting product mixture was treated with acetic acid, prior to purification using reverse-phase HPLC, to yield sar–dbm (3) in 8% yield. Similarly, dfo (4) was reacted with dibromomaleimide-*N*-glycine (5) and EEDQ in dimethyl sulfoxide, followed by purification using reverse-phase HPLC to give dfo–dbm (6, Scheme 2) in 22% yield.

The inclusion of a C_6 aliphatic linker group in sar–dbm aided reverse-phase HPLC purification and isolation of sar–dbm: the sar chelator motif is very hydrophilic and is poorly retained on reverse-phase columns in the absence of hydrophobic appendages. In contrast, the dfo chelator had sufficient adsorption on reverse-phase stationary phases, and so a C_2 linker group, which has demonstrated improved conjugation properties relative to a C_6 linker (*vide infra*),³⁴ was utilized.

The yield of sar–dbm (8%) was significantly lower than the yield of dfo–dbm (22%). We attribute this to the lower

reactivity of the apical primary amine groups of $(\text{NH}_2)_2\text{sar}$ and its complexes relative to other primary amines, possibly due to steric encumbrance of the macrobicyclic hexaamine rings. Previously reported syntheses, in which $(\text{NH}_2)_2\text{sar}$ complexes have been reacted with electrophilic motifs, have required significant heating.^{37–40} Here, the reaction was not heated, as the dibromomaleimide group is sensitive to heat. Although the yield of sar–dbm was low under these reaction conditions, it could be obtained in a one-pot synthesis from $[\text{Mg}((\text{NH}_2)_2\text{sar})]^{2+}$.

The compound sar–dbm₂, in which both apical primary amines of $(\text{NH}_2)_2\text{sar}$ react with 2 equiv of 3,4-dibromomaleimide-*N*-hexanoic acid to yield a sarcophagine chelator containing two dibromomaleimide groups, was also observed. Although we have detected and even isolated small amounts of sar–dbm₂ and its Mg^{2+} complex, $[\text{Mg}(\text{sar}-\text{dbm}_2)]^{2+}$, analyses of crude reaction mixtures revealed that it was present in low yield (see the Supporting Information).

■ RADIOLABELED SAR–DTM–TRASTUZUMAB AND DFO–DTM–TRASTUZUMAB

The monoclonal antibody trastuzumab was chosen as a model antibody for assessing conjugation of sar–dbm and dfo–dbm: trastuzumab targets the human epidermal growth factor receptor 2 (HER2) and is widely used for treatment of

HER2-positive breast cancer. The four solvent-accessible interstrand disulfide bonds of trastuzumab were reduced with tris(2-carboxyethyl)phosphine (TCEP), the reduced antibody was reacted with 8 mol equiv of either sar-dbm or dfo-dbm at pH 8.5, and the conjugation reactions were monitored over time by SDS-PAGE (*vide infra*). These reaction conditions were adapted from well-established methods for coupling drug-dibromomaleimide or fluorophore-dibromomaleimides to IgG1 constructs.^{31,34,41} Notably, increasing the molar equivalents of chelator-dbm relative to the trastuzumab antibody, for example, from 8 mol equiv of sar-dbm to either 16 or 24 mol equiv of sar-dbm, did not significantly affect the efficacy of the conjugation reaction (Figure S1).

For reaction of either sar-dbm or dfo-dbm with reduced trastuzumab, thiol substitution to give dithiomaleimide conjugates proceeded rapidly (within 5 min). Subsequent hydrolysis of the dithiomaleimide to the dithiomaleamic acid (dtm) was achieved by further incubation of the reaction solutions at pH 8.5 for 2–48 h (Scheme 3). Prior studies on this class of compounds have shown that dithiomaleimide hydrolysis proceeds faster in the presence of electron-withdrawing imide substituents: significantly, the proximity of the amide bond to the maleimide motif increases the rate of hydrolysis.³⁴ For dfo-dtm-trastuzumab, with a C₂ glycine-derived linker, hydrolysis (as monitored by SDS-PAGE) was complete within 2 h (Figure S2). The sar-dtm-sarcophagine conjugate, with a longer C₆ aminohexanoate linker, was incubated for 48 h at 37 °C to ensure complete hydrolysis (Figure S1).

As expected, the SDS-PAGE of TCEP-reduced trastuzumab showed the presence of separate heavy and light chains under the denaturing analysis conditions (Figure 1, lane C). Following dithiol rebridging reactions with chelator-dbm (for example sar-dbm, Figure 1, lane D, and dfo-dbm, Figure 1, lane G) and subsequent hydrolysis, the two major products observed corresponded to the “full antibody”, in which the maleamic acid groups bridge *inter* chain dithiols at the hinge region, and the “half antibody”, in which the maleamic acid groups bridge *intra* chain dithiols at the hinge region. The “full antibody” rebridging pattern mirrors that of the native trastuzumab antibody. The “half antibody” corresponds to a covalently linked single heavy chain + single light chain and is typical of this type of reaction.^{31,34} It arises as a result of “disulfide scrambling” and is only observed by harsh denaturing analysis. Less intense bands, corresponding to antibody heavy chains and light chains that remained “unbridged”, were also discernible, and these were more prominent for sar-dtm-trastuzumab than dfo-dtm-trastuzumab.

The same conjugation products were also analyzed using *reducing* SDS-PAGE conditions: dithiothreitol (DTT) was incubated with the antibody conjugate prior to running the SDS-PAGE. The same two “full antibody” and “half antibody bands” were observed (Figure 1, lanes E and H), indicating that (i) the conjugation and subsequent hydrolysis reactions to yield maleamic acid-conjugated motifs were complete, and (ii) the rebridged products were stable in the presence of excess thiols, consistent with prior reports detailing the high stability of such conjugates.^{31,34}

The bioconjugates sar-dtm-trastuzumab and dfo-dtm-trastuzumab were also characterized by ESI-HRMS, which showed that both sar-dtm-trastuzumab and dfo-dtm-trastuzumab contain two–four copies of each chelator. The

most intense signals in the deconvoluted ESI-HRMS of trastuzumab conjugates (Tables 1, S2, Figures S6, S7)

Table 1. ESI-MS Data for Chelator–Trastuzumab Conjugates

half antibody conjugate ^a	<i>m/z</i> (observed)	<i>m/z</i> (calculated)
sar-dtm-trastuzumab		
HL + 1 sar	73125	73116
HL + 2 sar	73647	73639
dfo-dtm-trastuzumab		
HL + 1 dfo		
HL + 2 dfo	74015	74017

^aAbbreviation: HL = heavy chain + light chain (i.e., half antibody).

corresponded to “half antibody”. Signals corresponding to “full antibody” were weak, which is a common feature of such immunoconjugates as the smaller fragments are always detected with greater relative intensity. Additionally, all deconvoluted “half antibody” and “full antibody” signals were significantly broader than that observed for similarly prepared and characterized alkyne–trastuzumab immunoconjugates.^{34,42} We attribute this peak broadening to sar-dtm-trastuzumab and dfo-dtm-trastuzumab readily complexing trace metal ions: we have previously observed trace metal binding to chelator conjugates in our mass spectrometric analyses.^{43,44}

The combination of SDS-PAGE and ESI-HRMS data indicates that the new immunoconjugates consist of a heterogeneous mixture, comprised of predominantly “full antibody” and “half antibody” constructs (with low amounts of conjugate in which some heavy and light chains remain “unbridged”), and contain two–four chelators per antibody. This heterogeneity, and in particular the presence of both “full antibody” and “half antibody” constructs, is unlikely to significantly modify the immunoconjugates’ affinity for the target HER2 receptor or their biodistribution *in vivo*: we have previously shown that mixtures containing similar “disulfide-scrambled” trastuzumab species exhibit comparable antigen-binding and Fc-binding properties to native trastuzumab.⁴²

The immunoconjugate sar-dtm-trastuzumab was radiolabeled with [⁶⁴Cu]Cu²⁺ at ambient temperature and pH 7 in 5 min, by addition of a solution of [⁶⁴Cu]Cu²⁺ (5 MBq, 30 μL, 0.1 M NH₄OAc) to the immunoconjugate (9.43 μL, 100 μg, 0.1 M NH₄OAc), and analyzed by size exclusion (SE) HPLC. In SE-HPLC, a PBS mobile phase containing ethylenediamine tetraacetate (EDTA) was used, and under these conditions, [⁶⁴Cu]Cu-sar-dtm-trastuzumab eluted at 7.8 min (Figure 2a, bottom trace) and unreacted [⁶⁴Cu]Cu²⁺ eluted at 11.6 min as [⁶⁴Cu][Cu(EDTA)]²⁻. This reaction provided [⁶⁴Cu]Cu-sar-dtm-trastuzumab in near quantitative radiochemical yield (>98%). Similarly, dfo-dtm-trastuzumab (10 μL, 72.5 μg, 0.1 M NH₄OAc) was radiolabeled with [⁸⁹Zr]Zr⁴⁺ (0.1 MBq, 5 μL, 0.2 M HEPES) at room temperature and pH 7 in 10 min, to yield [⁸⁹Zr]Zr-dfo-dtm-trastuzumab in 99% radiochemical yield (Figure 2b, bottom trace). In contrast, when a sample of unmodified trastuzumab was reacted with either [⁶⁴Cu]Cu²⁺ or [⁸⁹Zr]Zr⁴⁺, <2% of radioactivity was associated with the antibody, with the majority of unreacted radiometal eluting at 11.6 min (Figure 2a,b).

[⁶⁴Cu]Cu-sar-dtm-trastuzumab and [⁸⁹Zr]Zr-dfo-dtm-trastuzumab were additionally analyzed by SDS-PAGE with bright field imaging and autoradiography: a radioactivity signal from radiolabeled immunoconjugates was coincident with

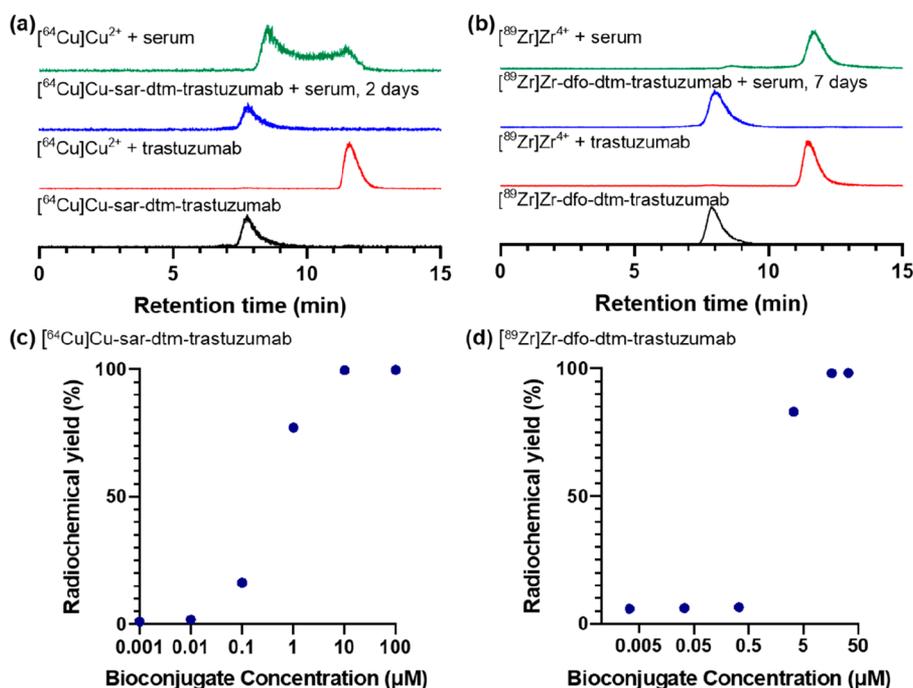


Figure 2. (a) SE-HPLC radiochromatograms of $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$ (black), $[^{64}\text{Cu}]\text{Cu}^{2+}$ with unmodified trastuzumab (red), $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$ incubated in serum for 2 days (blue), and $[^{64}\text{Cu}]\text{Cu}^{2+}$ incubated in serum (green). (b) SEC-HPLC radiochromatograms of $[^{89}\text{Zr}]\text{Zr-dfo-dtm-trastuzumab}$ (black), $[^{89}\text{Zr}]\text{Zr}^{4+}$ with unmodified trastuzumab (red), $[^{89}\text{Zr}]\text{Zr-dfo-dtm-trastuzumab}$ incubated in serum for 7 days (blue), and $[^{89}\text{Zr}]\text{Zr}^{4+}$ incubated in serum (green). (c) Radiochemical yields for the reaction of $[^{64}\text{Cu}]\text{Cu}^{2+}$ with sar-dtm-trastuzumab at different concentrations (100 μM , 10 μM , 1 μM , 100 nM, 10 nM, and 1 nM) of immunoconjugate at ambient temperature for 5 min. (d) Radiochemical yields for the reaction of $[^{89}\text{Zr}]\text{Zr}^{4+}$ with dfo-dtm-trastuzumab at different concentrations (33 μM , 16.7 μM , 3.3 μM , 333 nM, 33 nM, and 3 nM) of immunoconjugate at ambient temperature for 10 min.

stained protein bands (Figure 1, lanes F and I). For $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$, 41.1% of ^{64}Cu signal was associated with “full antibody”, 33.4% was associated with “half antibody”, 14.5% was associated with single heavy chain fragments, and 10.6% was associated with single light chain fragments. For $[^{89}\text{Zr}]\text{Zr-dfo-dtm-trastuzumab}$, 54.4% of ^{89}Zr signal was associated with “full antibody”, 34.9% was associated with “half antibody”, 7.7% was associated with single heavy chain fragments, and 3.0% was associated with single light chain fragments. The comparatively lower amount of radiolabeled single heavy chain and single light chain fragments for $[^{89}\text{Zr}]\text{Zr-dfo-dtm-trastuzumab}$ relative to $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$ suggested that dithiol rebridging was more efficient for dfo-dtm than sar-dtm.

To assess the serum stability of $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$ and $[^{89}\text{Zr}]\text{Zr-dfo-dtm-trastuzumab}$ *ex vivo*, the radiolabeled immunoconjugates were incubated in human serum at 37 °C. $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$ and $[^{89}\text{Zr}]\text{Zr-dfo-dtm-trastuzumab}$ both exhibited high stability in the presence of serum proteins: SE-HPLC radiochromatographic analysis indicated that >99% of $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$ remained intact after 2 days incubation in serum and >99% of $[^{89}\text{Zr}]\text{Zr-dfo-dtm-trastuzumab}$ remained intact after 7 days incubation in serum (Figure 2 a,b, S8, S9). In contrast, when $[^{64}\text{Cu}]\text{Cu}^{2+}$ was incubated in serum, SE-HPLC analysis showed that the majority of $[^{64}\text{Cu}]\text{Cu}^{2+}$ was complexed by a serum protein (retention time of 8.5 min) or low molecular weight species (retention time of 11.6 min). When $[^{89}\text{Zr}]\text{Zr}^{4+}$ was incubated in serum, the majority of radioactivity was associated with low molecular weight species (retention time of 11.8 min).

For preparations of radiopharmaceuticals based on chelator–protein bioconjugates, it is important that the amount of the precursor bioconjugate is relatively low. Typically, unlabeled immunoconjugate is not separated from the radiolabeled conjugate prior to *in vivo* administration, and high concentrations of unlabeled bioconjugate lead to *in vivo* receptor “blocking”, compromising PET image contrast. Furthermore, the ability to radiolabel in near quantitative radiochemical yields obviates purification steps. To assess the ability of sar-dtm-trastuzumab or dfo-dtm-trastuzumab to complex $[^{64}\text{Cu}]\text{Cu}^{2+}$ or $[^{89}\text{Zr}]\text{Zr}^{4+}$ respectively, solutions containing radiometal were added to increasingly dilute solutions of immunoconjugate (Figure 2c,d). Near quantitative radiochemical yields (>99%) of $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$ were achieved at immunoconjugate concentrations as low as 10 μM , pH 7 and ambient temperature, with only 5 min reaction time. At a lower sar-dtm-trastuzumab concentration of 1 μM , a high radiochemical yield of 77% was still obtained. Near quantitative radiochemical yields (>99%) of $[^{89}\text{Zr}]\text{Zr-dfo-dtm-trastuzumab}$ were achieved at 16 μM of dfo-dtm-trastuzumab at pH 7, ambient temperature and only 10 min reaction time. At a lower concentration of 3 μM , a relatively high radiochemical yield of 83% was still observed.

■ IN VITRO AND IN VIVO CHARACTERIZATION OF $[^{64}\text{Cu}]\text{Cu-SAR-DTM-TRASTUZUMAB}$

The binding of $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$ to its target receptor, HER2, was evaluated in HER2-positive human ovarian cancer cells (SKOV3 cells). $[^{64}\text{Cu}]\text{Cu-sar-dtm-trastuzumab}$ retained specificity for HER2 receptor: the radiotracer showed uptake in SKOV3 cells, with uptake

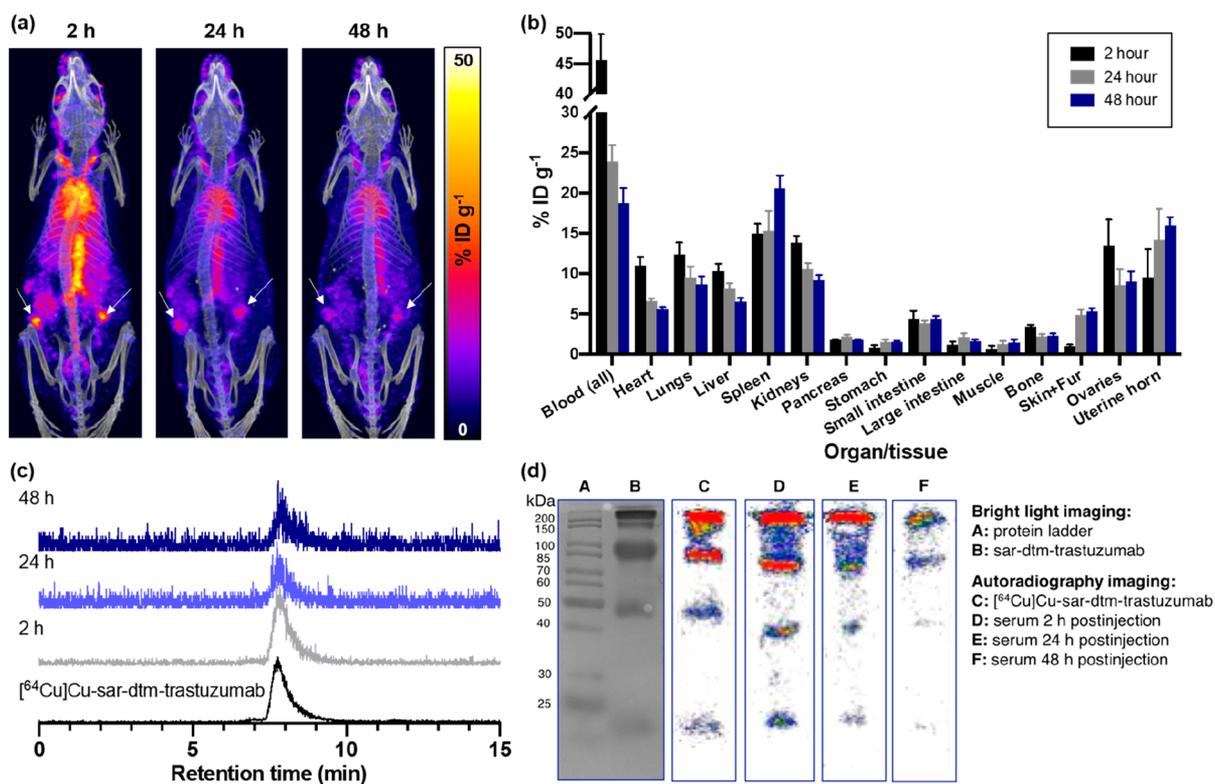


Figure 3. (a) PET/CT maximum intensity projections of a healthy female NOD scid gamma mouse administered [⁶⁴Cu]Cu-sar-dtm-trastuzumab, at 2, 24, and 48 h postinjection. White arrows indicate ovaries that are positive for HER2. (b) *Ex vivo* biodistribution of [⁶⁴Cu]Cu-sar-dtm-trastuzumab ($n = 4$ mice per time point). Error bars correspond to standard deviation. (c) SE-HPLC analysis of *ex vivo* serum samples shows a single radioactive signal in each chromatogram, with the retention time of each signal corresponding to [⁶⁴Cu]Cu-sar-dtm-trastuzumab. (d) SDS-PAGE of *ex vivo* serum samples (bright light and autoradiography imaging) shows radioactive bands corresponding to [⁶⁴Cu]Cu-sar-dtm-trastuzumab. The full gels are included in Figures S12, S13.

inhibited by increasing concentrations of unmodified trastuzumab (Figure S10).

We have previously assessed *in vivo* stability of radiometal-chelator complexes by quantifying the biodistribution of their IgG-based immunoconjugates in healthy mice over an extended period of time.^{3,19} In healthy mice, antibodies such as trastuzumab have long blood half-lives in the absence of diseased tissues/tumors that are positive for the target receptor. Here, *in vivo* studies in healthy animals aimed to assess the biodistribution and stability of [⁶⁴Cu]Cu-sar-dtm-trastuzumab *in vivo*. [⁶⁴Cu]Cu-sar-dtm-trastuzumab (1.0–2.5 MBq) was administered intravenously (via tail vein) to NOD scid gamma female mice. At 2 h, 1 day, and 2 days postinjection, PET/CT images were acquired, mice were culled, and organs and serum samples were collected for *ex vivo* analysis (Figures 3, S11).

PET/CT imaging and *ex vivo* biodistribution (Figures 3a,b, S11) showed that significant ⁶⁴Cu radioactivity remained in blood circulation over 1–2 days postinjection, although as typically observed, this activity decreased from $45.6 \pm 4.3\%ID$ (percentage injected dose) g^{-1} at 2 h postinjection to $18.7 \pm 1.9\%ID$ g^{-1} at 2 days postinjection. Importantly, ⁶⁴Cu radioactivity in the liver did not increase from 2 h to 2 days postinjection. Prior studies^{3,45} have shown that loss of [⁶⁴Cu]Cu from a bioconjugate (likely via transchelation to endogenous proteins including albumin in blood⁴⁶ and superoxide dismutase⁴⁷ and ceruloplasmin⁴⁸ in the liver) results in increased accumulation of [⁶⁴Cu]Cu in the liver over time. Our biodistribution data is consistent with prior reports

that detail exceptional stability of [⁶⁴Cu][Cu(sar)]²⁺ conjugates (both peptide and protein conjugates) *in vivo*.^{3,45}

Radioactivity uptake and retention in healthy HER2-expressing tissue also indicated that [⁶⁴Cu]Cu-sar-dtm-trastuzumab retains affinity for its target HER2 receptor (Figures 3a,b, S11). Notably, skin, uterine horn, and ovaries—healthy tissues that are known to express HER2^{49,50}—showed either increased uptake or persistent retention of ⁶⁴Cu radioactivity over the course of the study. For example, radioactivity uptake in skin increased from $1.0 \pm 0.2\%ID$ g^{-1} at 2 h postinjection to $4.9 \pm 0.7\%ID$ g^{-1} at 1 day postinjection ($p = 3.1 \times 10^{-5}$); at 2 days postinjection, skin samples showed retention of ⁶⁴Cu radioactivity ($5.3 \pm 0.4\%ID$ g^{-1}).

Serum samples were obtained from mice administered [⁶⁴Cu]Cu-sar-dtm-trastuzumab at 2 h, 1 day, and 2 days postinjection and analyzed by SE-HPLC and SDS-PAGE. In each SE-HPLC chromatogram, only a single signal was observed, with a retention time matching that of [⁶⁴Cu]Cu-sar-dtm-trastuzumab (Figure 3c). Serum samples were also subjected to SDS-PAGE separation, followed by autoradiography imaging, which revealed radioactive bands (Figure 3d, lanes D–F) that corresponded to denatured fragments of both unlabeled sar-dtm-trastuzumab (Figure 3d, lane B) and [⁶⁴Cu]Cu-sar-dtm-trastuzumab (Figure 3d, lane C). Significantly, neither SE-HPLC nor SDS-PAGE analyses revealed any radiolabeled species with molecular weights corresponding to serum albumin (with a molecular weight of 66.5 kDa), which is known to (i) react with maleimide derivatives released *in vivo* as a result of intramolecular retro-Michael reactions of

bioconjugates, resulting in transfer of maleimide groups and their cargo from bioconjugates to albumin,^{21–24} and additionally, (ii) compete with [⁶⁴Cu]Cu-radiolabeled chelator conjugates of peptides and proteins for [⁶⁴Cu]Cu²⁺ binding, resulting in release of [⁶⁴Cu]Cu from chelator conjugates.⁴⁶ In short, our experiments evidence the high stability of [⁶⁴Cu]Cu-sar-dtm-trastuzumab *in vivo*.

Concluding Remarks: Usability of Chelators Containing Dibromomaleimide Groups for Site-Specific Radiolabeling of an IgG Antibody. The majority of chelator-antibody conjugates are prepared from chelators containing reactive electrophilic groups that react with solvent accessible primary amines of lysine side chains. While these conjugation protocols are very simple to implement (even for researchers inexperienced in protein chemistry), such methods mostly lead to heterogeneous mixtures of chelator-protein conjugates that can exhibit suboptimal pharmacokinetics and decreased affinity for target receptors.^{6–8} Immunoconjugates containing conventional maleimide groups can enable site-specific attachment to reduced thiol groups of disulfide bonds of antibody derivatives, but the resulting conjugates undergo retro-Michael reactions in the biological milieu, resulting in loss of radioactive cargo.^{21–24} Many new chemical innovations in site-specific modification of antibodies have been adapted to stably incorporate chelators into antibodies and their derivatives;^{9–14} however, these methods involve enzymatic reactions, engineering of specific peptide sequences into the antibody and/or multistep procedures, and are therefore not simple to implement and tend to require extensive optimization for them to work reliably.

Dibromomaleimide chemistry is superbly suited to simple, stable, and site-specific incorporation of radionuclides into antibodies for receptor-targeted molecular imaging. We have prepared dibromomaleimide derivatives of (i) a dfo chelator, commonly used for [⁸⁹Zr]Zr⁴⁺ radiolabeling of antibodies, and (ii) a sarcophagine chelator, which is considered state-of-the-art for [⁶⁴Cu]Cu²⁺ radiolabeling of proteins and peptides, and successfully incorporated both new bifunctional chelators into the HER2-targeted IgG antibody, trastuzumab. These are the first examples of radiometal-labeled chelator-antibody conjugates that have been prepared using dibromomaleimide technology, enabling site-specific attachment of two different chelators to the IgG trastuzumab antibody. Importantly, the methods we describe are simple to implement and produce chelator-antibody conjugates that can be readily radiolabeled with the PET radiometals [⁸⁹Zr]Zr⁴⁺ and [⁶⁴Cu]Cu²⁺ in near-quantitative radiochemical yields at ambient temperature. We have evidenced the high stability of [⁶⁴Cu]Cu-sar-dtm-trastuzumab both in serum and *in vivo*: it is stable to both demetalation of [⁶⁴Cu]Cu, consistent with the known high stability of [⁶⁴Cu][Cu(sar)]²⁺ complexes, and transfer of the [⁶⁴Cu][Cu(sar)]²⁺ to endogenous thiols, consistent with the reported stability of dithiomaleamic acid groups. Our future efforts will compare the biological properties of these site-specifically radiolabeled immunoconjugates with radiolabeled immunoconjugates modified using either conventional maleimide conjugation chemistry or non-site-specific (stochastic) conjugation chemistry.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.0c00710>.

Experimental descriptions, full SDS-PAGE data, ESI-MS, NMR, HPLC chromatograms, PET/CT image analysis, cell binding study data (PDF)

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Notes

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