Modular Strategy for the Construction of Radiometalated Antibodies for Positron Emission Tomography Based on Inverse Electron Demand Diels—Alder Click Chemistry

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Supporting Information

ABSTRACT: A modular system for the construction of radiometalated antibodies was developed based on the bioorthogonal cycloaddition reaction between 3-(4-benzylamino)-1,2,4,5-tetrazine and the strained dienophile norbornene. The well-characterized, HER2-specific antibody trastuzumab and the positron emitting radioisotopes $^{64}$Cu and $^{89}$Zr were employed as a model system. The antibody was first covalently coupled to norbornene, and this stock of norbornene-modified antibody was then reacted with tetrazines bearing the chelators 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or desferrioxamine (DFO) and subsequently radiometalated with $^{64}$Cu and $^{89}$Zr, respectively. The modification strategy is simple and robust, and the resultant radiometalated constructs were obtained in high specific activity (2.7⋯5.3 mCi/mg). For a given initial stoichiometric ratio of norbornene to antibody, the $^{64}$Cu-DOTA- and $^{89}$Zr-DFO-based probes were shown to be nearly identical in terms of stability, the number of chelates per antibody, and immunoreactivity (>93% in all cases). In vivo PET imaging and acute biodistribution experiments revealed significant, specific uptake of the $^{64}$Cu- and $^{89}$Zr-trastuzumab bioconjugates in HER2-positive BT-474 xenografts, with little background uptake in HER2-negative MDA-MB-468 xenografts or other tissues. This modular system—one in which the divergent point is a single covalently modified antibody stock that can be reacted selectively with various chelators—will allow for both greater versatility and more facile cross-comparisons in the development of antibody-based radiopharmaceuticals.

INTRODUCTION

Over the past two decades, radiopharmaceuticals based on antibodies have assumed an increasingly prominent role in both diagnostic and therapeutic nuclear medicine. This trend is particularly evident in the field of positron emission tomography (PET), in which a wide variety of effective antibody-based radiotracers have been developed against an array of cancer biomarkers.¹⁻³ Indeed, while some promising imaging agents have been labeled with long-lived nonmetallic radionuclides such as $^{124}$I, the majority of antibody-based PET bioconjugates have employed positron-emitting radioisotopes, including $^{64}$Cu, $^{86}$Y, and, most recently, $^{89}$Zr.⁴⁻⁸ In these systems, radiometals offer significant advantages over their nonmetallic cousins, most notably decay characteristics that result in high image quality, radioactive half-lives that complement the biological half-lives of the antibody vectors, and enhanced control and ease of radiolabeling through the use of chelating moieties.

Despite their benefits, however, these chelating moieties are the source of a somewhat confounding issue in the study of radiometalated antibodies. Put simply, different radiometals require different chelators. For example, the small, hard $^{89}$Zr$^{4+}$ cation shows very high affinity for the multiple oxygen donors of the chelator desferrioxamine (DFO), while the larger and softer $^{64}$Cu$^{2+}$ cation exhibits higher thermodynamic and kinetic stability when bound to chelators bearing nitrogen donors in addition to oxygen donors, for example, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyldiacetic acid (CB-TE2A).⁶⁻⁹ Further, different chelators often require dramatically different synthetic strategies for antibody couplings.¹⁰ In an isolated case of one antibody and one radiometal, these facts do not present a problem. However, they do create a significant obstacle to the versatility of radiometalated bioconjugates. To wit, given a particular monoclonal antibody, the development of a $^{64}$Cu-CB-TE2A-mAb conjugate for PET, a $^{89}$Zr-DFO-mAb conjugate for PET, and a $^{225}$Ac-DOTA-mAb conjugate for therapy would require three different routes.

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for antibody modification. Not only would this require additional time to develop and optimize each pathway, but the disparate routes would also mandate differing reaction conditions for each antibody, opening the door for differences in immunoreactivity and chelator/antibody ratio and ultimately making meaningful comparisons among the various radiopharmaceuticals more difficult. Consequently, a modular system—one in which the divergent point is a single covalently modified antibody stock that can be reacted selectively with various chelators—would resolve these issues and allow for more versatility and cross-comparisons in the development of antibody-based radiopharmaceuticals.

The chemical requirements of such a modular system—selectivity, biocompatibility, biorthogonality—make it an almost perfect application for the use of click chemistry. Coined by K. Barry Sharpless, the term “click chemistry” broadly defines a group of chemical reactions by which two molecular components can be joined via a selective, rapid, clean, bioorthogonal, and biocompatible ligation.11–13 By far, the most popular example of click chemistry is the Cu(I)-catalyzed [3 + 2] Huisgen cycloaddition between an azide and alkyne.14 This reaction has already been widely employed in the development of radiotracers, particularly 18F-based PET probes.15–18 The application of this technology to radiometal-based probes has lagged behind, however, most likely due to concerns over metal contamination by the catalyst itself, though “clickable” chelators based on both the Cu(I)-catalyzed reaction and other Cu(I)-free systems have become more common in the literature in recent years.19–22 Very recently, another promising “click” variant has come to light: the inverse electron demand Diels–Alder reaction between a tetrazine moiety and a strained alkene dienophile (Figure 1).23–25 Like other click reactions, the ligation is selective, fast, biocompatible, and bioorthogonal, and unlike many Diels–Alder reactions, the coupling is irreversible, forming stable pyrazidine products after the retro-Diels–Alder release of dinitrogen from the reaction intermediate. A number of different tetrazine–strained alkene pairs have been explored for the reaction, though the combination of 3-(4-benzylamino)-1,2,4,5-tetrazine (Tz) and either norbornene- or trans-cyclooctene-derivatives seems well-suited for biological applications. To date, the ligation has been employed in a variety of settings: the modification of oligonucleotides;26 fluorescence imaging with small molecules, antibodies, and nanoparticles;27,28 SPECT imaging with antibodies;29 and 18F-PET imaging with peptides.30,31 However, to the best of the authors’ knowledge, no application of this technology to positron-emitting radiometals has yet been made.

Herein, we report the development of a modular strategy for the construction of radiolabeled antibodies using the tetrazine–norbornene click reaction. The synthetic pathway involves three simple steps: (1) creation of a common stock of norbornene-modified antibody via peptide coupling; (2) ligation of a chelator-modified tetrazine moiety to the norbornene-modified antibody; and (3) radiolabeling of the resultant construct (Figure 2). For this proof of concept investigation, we have chosen the positron-emitting radiometals 64Cu and 89Zr, the chelators DOTA and DFO, and the antibody trastuzumab. 64Cu and 89Zr are the two most common radionuclides employed in antibody-based PET bi conjugates, and DOTA and DFO, respectively, are the most common chelators employed with these two metals.6,9,10,32–34

The antibody trastuzumab (Herceptin, Genentech), is an extremely well-characterized antibody specific to the human epidermal growth factor receptor 2 (HER2, also known as ERBB2). Overexpression of HER2 has been shown to be associated with augmented metastatic potential, increased tumor aggression, and poor prognosis for disease-free survival for patients with a variety of malignancies, most notably breast, ovarian, and colorectal cancer.35–38 Trastuzumab alone has been employed as a therapeutic agent, and conjugates of both the antibody and its derivative fragments have been synthesized bearing a wide variety of radionuclides—including 64Cu, 86Y, 111In, 124I, 99mTc, and 89Zr—for PET and SPECT imaging of HER2 expression.39–47 It is our hope that this modular methodology will aid in both the expansion of the comparative study of antibodies labeled with different radionuclides and the development of novel antibody-based radiopharmaceuticals. Since more and more antibodies and diphosphines are being developed, this modular approach will likely lead to the rapid development of many novel imaging agents.28 Importantly, while we have used positron-emitting radionuclides in this study due to our laboratory’s area of expertise, this modular system need not be applied only to PET radiometals but rather can be used across the spectrum of metallic radi nuclides, encompassing those employed for SPECT and radiotherapy as well.

**EXPERIMENTAL PROCEDURES**

**Materials.** All chemicals, unless otherwise noted, were acquired from Sigma-Aldrich (St. Louis, MO) and were used as received without further purification. All water employed was ultrapure (>18.2 MΩ cm−1 at 25 ºC, Milli-Q, Millipore, Billerica, MA), and was passed through a 10 cm column of Chelex resin (Bio-Rad Laboratories, Hercules, CA) before use. DMSO was of molecular biology grade (>99.9%; Sigma, D8418), and all other solvents were of the highest grade commercially available. 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid monohydroxysuccinimidyldote (DOTAL-NHS) was purchased from Macrocyclics Inc. (Dallas, TX). N-Succinyl-6-feroxamine B was prepared according to published procedures.48 All instruments were calibrated and maintained in accordance with standard quality-control procedures.49 UV–vis measurements were taken on a Cary 100 Bio UV–vis spectrophotometer. NMR spectroscopy was performed on a Bruker 500 MHz NMR with Topsis 2.1 software for spectrum analysis. HPLC was performed using a Shimadzu HPLC equipped with a C-18 reversed-phase column (Phenomenex Luna analytical 4.6 × 250 mm; Semi-Prep 21.2 × 100 mm, 5 μm, 1.0 or 6.0 mL/min), 2 LC-10AT pumps, a SPD-M10AVP photodiode array detector, and a gradient of 0:100 MeCN/H2O (both with 0.1% TFA) to 100:0 MeCN/H2O over 15 min.

64Cu was purchased from Washington University, St. Louis, where it was produced on the Washington University School of Medicine Cyclotron (model CS-15, Cyclotron Corp.) by the 64Ni(p,n)64Cu reaction and purified as previously described to...
yield \[^{64}\text{Cu}]\text{CuCl}_2\) with an effective specific activity of 200–400 mCi/\(\mu\)g (7.4–14.8 GBq/\(\mu\)g). \[^{89}\text{Zr}\] was produced at Memorial Sloan-Kettering Cancer Center on an EBCO TR19/9 variable-beam energy cyclotron (EBCO Industries Inc., British Columbia, Canada) via the \[^{89}\text{Y}(p, n)^{89}\text{Zr}\] reaction and purified in accordance with previously reported methods to yield \[^{89}\text{Zr}\] with a specific activity of 5.28–13.43 mCi/\(\mu\)g (195–497 MBq/\(\mu\)g). All buffers used for \[^{64}\text{Cu}\] and \[^{89}\text{Zr}\] labeling reactions were monitored using silica-gel impregnated glass-fiber instant thin layer chromatography paper (Pall Corp., East Hills, NY) and analyzed on a Bioscan AR-2000 radio-TLC plate reader using Winscan Radio-TLC software (Bioscan Inc., Washington, DC). Human breast cancer cell lines BT-474 and MDA-MB-468 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown by serial passage.

**Synthesis of 3-(4-Benzylamino)-1,2,4,5-tetrazine (Tz).** The protocol from Deveraj et al. was employed for the synthesis with slight modifications.\(^{24}\) 4-(Aminomethyl)-benzonitrile hydrochloride (0.84 g, 0.005 mol) formamidine acetate (2.08 g, 0.02 mol), and elemental sulfur (0.16 g, 0.005 mol) were added to a DMSO solution (3 mL). The DMSO solution was added to a second, premixed solution of benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP, 53 mg, 0.12 mmol) in DMSO (3 mL), and diisopropylethylamine (16 \(\mu\)L, 0.09 mmol) was added to this solution. After 15 min of stirring at RT, the pink DMSO solution was added to a second, premixed solution of \(N\)-succinyldeferoxamine B (60 mg, 0.09 mmol) and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP, 53 mg, 0.12 mmol) in DMSO (3 mL). The combined reaction was stirred overnight and subsequently purified via C\(_{18}\) cartridge (Waters C\(_{18}\) Sep-Pak, Waters Corp., Milford, MA) and semipreparative reverse-phase HPLC. The purified product was obtained in 50% yield (molecular weight = 852.9, 19 mg, 0.023 mmol). \(^{1}\)H NMR (500 MHz, DMSO-\(\text{d}_6\)), \(\delta\) ppm: 10.59 (s, 1H), 9.64 (s, 1H), 9.59 (s, 1H), 8.49 (m, 1H), 8.44 (d, 2H), 7.9–7.7 (m, 3H), 7.51 (d, 2H), 4.44 (d, 2H), 3.5–3.55 (m, 6H), 3.05–2.95 (m, 6H), 2.55 (t, 4H), 2.45–2.35 (m, 4H), 2.25 (t, 4H), 1.97 (s, 3H), 1.52–1.48 (m, 6H), 1.40–1.36 (m, 6H), 1.23–1.20 (m, 6H). ESI-MS: 831.5 \([M+H]^+\), 853.6 \([M+Na]^+\). HPLC \(t_R = 10.2\) min.

**Synthesis of 2,2',2''-(10-(2-(4-(1,2,4,5-Tetrazin-3-yl)benzylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclodecane-1,4,7-triyl)triacetic acid (Tz-DOTA).** 3-(4-Benzylamino)-1,2,4,5-tetrazine (20 mg, 0.12 mmol) was dissolved in PBS (5 mL, pH 8.5), and disopropylethylamine (40 \(\mu\)L, 0.24 mmol) was added.

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**Figure 2.** Schematic of a modular strategy for the construction of \[^{89}\text{Zr}\]- and \[^{64}\text{Cu}\]-modified antibody bioconjugates using the tetrazine-norbornene ligation.
to this solution. This solution was then added to solid DOTA-NHS (50 mg, 0.065 mmol), and the resultant solution was stirred overnight at room temperature. The reaction was subsequently purified via C18 cartridge (Waters C18 Sep-Pak, Waters Corp., Milford, MA) and semipreparative reverse-phase HPLC. The purified product was obtained in 62% yield (molecular weight = 573.6, 23 mg, 0.04 mmol). 1H NMR (500 MHz, DMSO-d6), δ ppm: 10.61 (s, 1H), 9.11 (br s, 1H), 8.50 (d, 2H), 7.62 (d, 2H), 4.50 (s, 2H), 4.42–4.38 (m, 4H), 3.65 (br s, 4H), 10.16 (s, 1H), 3.65–3.55 (m, 8H), 3.18–3.14 (m, 8H). ESI-MS: 574.5 [M+H]+, 596.1 [M+Na]+, 612.2 [M+K]+. HPLC tR = 8.1 min.

Antibody Modification. A protocol similar to that published by Devaraj et al. was employed for antibody modification. 24-5-Norbornene-2-carboxylic acid (40 mg, 0.29 mmol) was incubated with 1.3 equiv of disuccinimidyl carbonate (100 mg, 0.39 mmol) and 1 equiv of pyridine (23 mg, 0.29 mmol) in dry acetonitrile (3 mL) for 2 h at room temperature. After 2 h, the solvent was removed via rotary evaporation, and the crude norbornene-succinimidyl ester product was recovered. After Trastuzumab (purchased commercially as Herceptin, Genentech, San Francisco, CA) was purified using centrifugal filter units with a 30 000 molecular weight cutoff (Amicon Ultra 4 Centrifugal Filter Units, Millipore Corp., Billerica, MA) and phosphate buffered saline (PBS, pH 7.4) to remove filtration units, Millipore Corp., Billerica, MA) and phosphate buffered saline (PBS pH 7.4 at 4°C) and a 10-fold molar excess of either Tz-DOTA or Tz-DFO in 10 μL DSMSO (molar excess calculated based on initial norbornene reaction stoichiometry). The reaction was incubated at RT for 5 h and subsequently purified using centrifugal filtration to yield the completed DOTA- and DFO-modified antibodies. The final bioconjugates were stored in PBS pH 7.4 at 4°C.

Labeling of DOTA-T/N-trastuzumab with 64Cu. DOTA-T/N-trastuzumab (0.2–0.3 mg) was added to 200 μL labeling buffer (50 mM NH₄OAc, pH 5.5, though 50 mM NaOAc, pH 5.5 also is sufficient). 64CuCuCl₂ (29.6 μM, 0.04 mmol). 1H NMR (500 MHz, DMSO-d6), δ ppm: 10.61 (s, 1H), 9.11 (br s, 1H), 8.50 (d, 2H), 7.62 (d, 2H), 4.50 (s, 2H), 4.42–4.38 (m, 4H), 3.65 (br s, 4H), 10.16 (s, 1H), 3.65–3.55 (m, 8H), 3.18–3.14 (m, 8H). ESI-MS: 574.5 [M+H]+, 596.1 [M+Na]+, 612.2 [M+K]+. HPLC tR = 8.1 min.

Stability Measurements. The stability of the 64Cu-DOTA- and 89Zr-DFO-T/N-trastuzumab bioconjugates with respect to radiolabeling and loss of radioactivity from the antibody was investigated via incubation of the antibodies in human serum for 48 h (64Cu) or 7 d (89Zr) at room temperature and 37°C. The radiochemical purity of the antibodies was determined using radio-TLC with an eluent of 50 mM EDTA pH 5.0 (vide supra).

Cell Culture. Human breast cancer cell lines BT474 and MDA-MB-468 were obtained from the American Type Culture Collection (HTB-20 and HTB-132, respectively, ATCC, Bethesda, MD) and maintained in a 1:1 mixture of Dulbecco’s Modified Eagle medium: F-12 medium, supplemented with 10% heat-inactivated fetal calf serum (Omega Scientific, Tarzana, CA), 2.0 mM glutamine, nonessential amino acids, and 100 units/mL penicillin, and 100 units/mL streptomycin in a 37°C environment containing 5% CO₂. Cell lines were harvested and passaged weekly using a formulation of 0.25% trypsin/0.53 mM EDTA in Hank’s Buffered Salt Solution without calcium and magnesium.

Xenograft Models. All experiments were performed under an Institutional Animal Care and Use Committee-approved protocol, and the experiments followed institutional guidelines for the proper and humane use of animals in research. Six- to eight-week-old Athymic nu/nu female mice (NCRNU-M) were obtained from Taconic Farms Incorporated (Hudson, NY). Animals were
housed in ventilated cages, were given food and water ad libitum, and were allowed to acclimatize for approximately 1 week prior to treatment. Prior to tumor inoculation, mice were subcutaneously implanted with 0.72 mg 60 day release 17β-estradiol pellets (SE-121, Innovative Research of America, Sarasota, Florida) using a 10 gauge trocar. After several days, BT474 tumors were induced on the right shoulder by a subcutaneous injection of 3.0 × 10⁶ cells in a 100 μL cell suspension of a 1:1 mixture of fresh media/BD Matrigel (BD Biosciences, Bedford, MA). MDA-MB-468 tumors were induced on the left shoulder by a subcutaneous injection of 2.0 × 10⁶ cells in the same manner (the number of cells injected was varied as described to compensate for cell growth rates and thus provide approximately the same tumor size at the time of radiopharmaceutical injection).

Acute Biodistribution. Acute in vivo biodistribution studies were performed in order to evaluate the uptake of the ⁶⁴Cu-DOTA- and ⁸⁹Zr-DFO-conjugated antibodies in mice bearing bilateral, subcutaneous BT-474 and MDA-MB-468 tumors (100–150 mm³), 4 weeks postinoculation. Mice were randomized before the study and were warmed gently with a heat lamp for 5 min before administration of ⁶⁴Cu-DOTA-T/N-trastuzumab (0.74–1.14 MBq [20–30 μCi] in 200 μL 0.9% sterile saline) or ⁸⁹Zr-DFO-T/N-trastuzumab (0.56–0.74 MBq [15–20 μCi] in 200 μL 0.9% sterile saline) via intravenous tail vein injection (t = 0). Animals (n = 4 per group) were euthanized by CO₂(g) asphyxiation at 6, 12, 24, 36, 48, and 72 h (⁶⁴Cu) or 6, 24, 48, 72, 96, and 120 h (⁸⁹Zr). After asphyxiation, 13 organs (including both tumors) were removed, rinsed in water, dried in air for 5 min, weighed, and counted in a gamma counter calibrated for either ⁶⁴Cu or ⁸⁹Zr. Counts were converted into activity using a calibration curve generated from known standards. Count data were background- and decay-corrected to the time of injection, and the percent injected dose per gram (%ID/g) for each tissue sample was calculated by normalization to the total activity injected.

Small-Animal PET Imaging. PET imaging experiments were conducted on either a microPET Focus 120 (⁸⁹Zr) or a microPET R4 (⁶⁴Cu) rodent scanner (Concorde Microsystems).⁵⁶ Mice bearing bilateral, subcutaneous BT-474 (right shoulder) and MDA-MB-468 (left shoulder) tumors (100–150 mm³), 4 weeks postinoculation) were administered ⁶⁴Cu-DOTA-T/N-trastuzumab (11.1–12.9 MBq [300–345 μCi] in 200 μL 0.9% sterile saline) or ⁸⁹Zr-DFO-T/N-trastuzumab (10.7–11.8 MBq [290–320 μCi] in 200 μL 0.9% sterile saline) via intravenous tail vein injection (t = 0). Approximately 5 min prior to the acquisition of PET images, mice were anesthetized by inhalation of 2% isoflurane (Baxter Healthcare, Deerfield, IL)/oxygen gas mixture and placed on the scanner bed; anesthesia was maintained using 1% isoflurane/oxygen mixture. PET data for each mouse were recorded via static scans at various time points between 6 and 120 h. A minimum of 20 million coincident events were recorded using 1% isoflurane/gas mixture. PET data for each mouse were rebinned, and transverse images were reconstructed by filtered back-projection (FBP) into a 128 × 128 × 63 (0.72 × 0.72 × 1.3 mm³) matrix. The image data were normalized to correct for nonuniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay to the time of injection, but no attenuation, scatter, or partial-volume averaging correction was applied. The counting rates in the reconstructed images were converted to activity concentrations (percentage injected dose [%ID] per gram of tissue) by use of a system calibration factor derived from the imaging of a mouse-sized water-equivalent phantom containing ⁶⁴Cu or ⁸⁹Zr. Images were analyzed using ASIPro VM software (Concorde Microsystems).

Labeling Norbornene-Trastuzumab with [⁶⁴Cu]-Tz-DOTA. Tz-DOTA (5 μL of 1 mM solution in DMSO) was added to labeling buffer (50 mM NH₄OAc, pH 5.5), and [⁶⁴Cu]CuCl₂ (40.7–55.5 MBq [1100–1500 μCi]) in 0.1 M HCl were added to the reaction mixture. The resultant solution was incubated for 1 h at 85 °C, followed by purification via C₁₈ cartridge (Waters Sep-Pak, Milford, MA) and radiochemical purity analysis via analytical HPLC (tᵣ = 10 min). The purified, radiolabeled [⁶⁴Cu]-Tz-DOTA was then added to a solution of norbornene-modified trastuzumab (0.4 mg, initial reaction stoichiometry of 5:1 norbornene/mAb) in PBS pH 7.4. The reaction mixture was allowed to incubate at 37 °C for 3 h. After 3 h, the progress of the reaction was assayed with radio-TLC using an eluent of 50 mm EDTA pH 5.0, and the radiolabeled antibody was purified with centrifugal filtration using centrifugal filter units with a 30 000 molecular weight cutoff (Amicon Ultra 4 Centrifugal Filtration Units, Millipore Corp., Billerica, MA) and phosphate buffered saline (PBS, pH 7.4). The radiochemical purity of the final radiolabeled bioconjugate was assayed again by radio-TLC and was found to be >99% in all preparations. In the radio-TLC experiments, ⁶⁴Cu-DOTA-T/N-trastuzumab remains at the baseline, while ⁶⁴Cu⁺ ions, [⁶⁴Cu]⁻Cu-Tz-DOTA, and [⁶⁴Cu]⁺Cu-EDTA elute with the solvent front.

Statistical Analysis. Data were analyzed by the unpaired, two-tailed Student’s t test. Differences at the 95% confidence level (P < 0.05) were considered to be statistically significant.

RESULTS AND DISCUSSION

Chemical Synthesis. 3-(4-Benzylamino)-1,2,4,5-tetrazine (Tz) was successfully synthesized through the reaction of 4-(aminomethyl)-benzonitrile hydrochloride, formamidine acetate, and elemental sulfur to form a dihydrotetrazine intermediate (4-(1,2-dihydroy-1,2,4,5-tetrazin-3-yl)phenyl)methanamine, followed by oxidation with NaNO₂ to form the aromatic tetrazine product. A method similar to that published by Devaraj et al. was employed; however, a number of small changes—for example, the use of 1% HCl(aq) rather than acetic acid in an intermediate step—were made and were found to considerably raise yields from the reported 20% to 35–40%. The product was characterized via UV—vis, ¹H NMR, ¹³C NMR, and ESI-MS, and all data match that described in the original synthetic report.²⁴ Given the particularly promising nature of this cycloaddition reaction, the optimization of this synthesis was an important task. Tz was chosen as the particular tetrazine-based moiety for this line of experimentation due to its convenient, primary-amine coupling handle and its balance of reactivity and stability. To be sure, other tetrazine-based molecules with possible conjugation sites exist, but water instability (dimethyl 1,2,4,5-tetrazine-3,6-dicarboxylate), poor reactivity (1,2,4,5-tetrazine-3,6-diamine or 3,6-bis-(4-aminophenyl)-1,2,4,5-tetrazine), or instability (6-(6-pyrindin-2-yl)-1,2-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-amine) render them unsuitable to the development of a modular system such as this.²⁹ ⁵⁷–⁵⁹ Tz-DOTA and Tz-DFO (Scheme 1) were synthesized from Tz via simple peptide coupling reactions using the commercially available mono-NHS-ester of DOTA or N-succinimidyl ester of B and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), respectively.

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Scheme 1. Synthetic Route to Tz-DOTA and Tz-DFO

Table 1. Chemical and Biological Characterization Data for $^{64}$Cu-DOTA-T/N- and $^{89}$Zr-DFO-T/N-trastuzumab Bioconjugates

| radionuclide | chelator | initial Nor/mAb reaction stoichiometry | chelates/mAb$^a$ | specific activity (mCi/mg) | immunoreactive fraction$^b$ | stability$^c$ |
|--------------|----------|--------------------------------------|------------------|----------------------------|-----------------------------|---------------|
| $^{64}$Cu    | DOTA     | 1.5                                   | 1.0 ± 0.2        | 3.2 ± 0.4                  | 0.96 ± 0.05                 | >98%          |
|              |          | 3                                     | 2.3 ± 0.4        | 3.1 ± 0.2                  | 0.95 ± 0.03                 | >96%          |
|              |          | 5                                     | 3.7 ± 0.7        | 5.3 ± 0.5                  | 0.94 ± 0.02                 | >96%          |
| $^{89}$Zr    | DFO      | 1.5                                   | 1.1 ± 0.3        | 2.7 ± 0.2                  | 0.96 ± 0.03                 | >98%          |
|              |          | 3                                     | 2.2 ± 0.3        | 2.9 ± 0.3                  | 0.96 ± 0.04                 | >98%          |
|              |          | 5                                     | 3.8 ± 0.9        | 4.3 ± 0.4                  | 0.93 ± 0.05                 | >97%          |

$^a$ n = 3 for all experiments presented. $^b$ Determined prior to in vivo experimentation. $^c$ Calculated for incubation in human serum at 37 °C for 48 h (Cu) or 7 d (Zr).

Upon synthesis, both molecules were purified via reversed-phase HPLC and fully characterized by UV—vis, $^1$H NMR, $^{13}$C NMR, and ESI-MS. Importantly, Tz-DOTA exhibits high water solubility, but Tz-DFO does not. Consequently, DMSO was used as the stock solvent and delivery vehicle for both Tz-DOTA and Tz-DFO throughout the investigation in order to ensure that the antibodies in different branches of the modular pathway were exposed to exactly the same reaction conditions. Indeed, given the ease of synthesis of both Tz-DOTA and Tz-DFO, it is easy to envision the creation of a complete library of tetrazine-modified chelators—ranging from Tz-AmBaSar to Tz-HBED to Tz-DTPA—in order to maximize the utility and versatility of this modular construction strategy.

Antibody Modification, Radiolabeling, and Characterization. The radiolabeled trastuzumab bioconjugates were constructed via a modular three-step procedure (Figure 2). A common stock of norbornene-modified mAb was first produced via the room-temperature aqueous coupling of an NHS-ester of 5-norbornene-2-carboxylic acid with the exposed lysines of trastuzumab. After purification, the resultant norbornene-modified antibodies were then incubated for $5$ h at room temperature with a 10-fold excess (based on norbornene loading) of the appropriate tetrazine-modified chelator—Tz-DOTA for $^{64}$Cu or Tz-DFO for $^{89}$Zr—and purified via centrifugal filtration or size exclusion chromatography. The bioconjugates were radiometalated with $^{64}$Cu or $^{89}$Zr at room temperature under either acidic (pH 5.5) or basic (pH 7.2–8.5) conditions, respectively. The crude radiochemical yields varied according to the initial norbornene loading of the antibody; however, after purification via centrifugal filtration, the $^{64}$Cu-DOTA- or $^{89}$Zr-DFO-T/N-trastuzumab conjugates were isolated with RCP >99% ($n = 3$ for each construct). The modification and radiolabeling strategy is simple, robust, and relatively rapid, and no antibody aggregation or precipitation issues were observed. Unlike other methods for the modification of mAbs with DOTA or DFO, overnight incubations, wide swings in buffer pH, and temperatures over room temperature are not required.60–64 Importantly, we also observed that the tetrazine-norbornene ligation and subsequent radiolabeling proceeded almost identically whether performed the day of norbornene modification of the antibody or four weeks later (and likely after much longer periods of time, provided the antibody is stored at 4 °C). The radiolabeling of the DOTA- and DFO-modified trastuzumab conjugates is likewise robust, with the reaction providing similar yields with freshly prepared or four-week-old mAbs.

A number of chemical and in vitro tests were performed in order to characterize the chelator-modified and radiolabeled antibody constructs. Three different initial reaction stoichiometries of norbornene:mAb—1:5:1, 3:1, and 5:1—were employed to investigate the effect of different chelator loadings on the performance of the antibody. After the ligation of the variably norbornene-loaded antibodies with either Tz-DOTA or Tz-DFO, radiometric isotopic dilution experiments were performed in order to determine the number of accessible chelates on each antibody. The results, shown in Table 1, clearly illustrate that increasing initial loadings of norbornene result in higher numbers of chelates per antibody. Given the quantitative nature of the tetrazine/norbornene ligation and the proximity of the number of chelates per antibody to the initial modification stoichiometry in each case, calculating the loading of norbornenes per antibody was deemed unnecessary. The combined yield of the modification and ligation reactions is relatively consistent across all three
stochiometries (~40–60%), and the results are generally consistent with antibody ligations using tetrazine/dienophile pairs reported by Devaraj et al., Haun et al., and Rossin et al.\textsuperscript{53,28,29} Just as importantly, the number of chelates per antibody is, within error, identical for both the DOTA-T/N-trastuzumab and DFO-T/N-trastuzumab conjugates, a critical facet for such a modular system. Not surprisingly, the varying chelate numbers also played a role in the specific activities obtained for each antibody. All of the antibody conjugates were labeled in high specific activity (>2.0 mCi/mg). Interestingly, the specific activities of both the \(^{64}\text{Cu}\)-DOTA-T/N-trastuzumab and \(^{89}\text{Zr}\)-DFO-T/N-trastuzumab conjugates only roughly correlate with the number of chelates per antibody: those for \(^{64}\text{Cu}\)-DOTA-T/N-trastuzumab range from 3.2 ± 0.4 mCi/mg to 5.3 ± 0.5 mCi/mg, though the specific activities for bioconjugates with initial nor/mAb ratios of 1.5:1 and 3:1 are within error of each other. Similarly, the specific activities of the \(^{89}\text{Zr}\)-DFO-T/N-trastuzumab conjugates range from 2.7 ± 0.2 mCi/mg to 4.3 ± 0.4 mCi/mg, but again, the specific activities of the two conjugates with fewer DFO/mAb are statistically identical. Given the different specific activities of the original radiometals, comparisons between the specific activities of the two types of construct have little merit; however, the specific activities obtained in this investigation are consistent with those reported for other \(^{64}\text{Cu}\)-DOTA-based and \(^{89}\text{Zr}\)-DFO-based antibody bioconjugates in the literature.\textsuperscript{5,10,34,42,45}

The immunoreactive fractions of the \(^{64}\text{Cu}\)-DOTA- and \(^{89}\text{Zr}\)-DFO-conjugates were determined via specific in vitro cellular association assays using the HER2/neu positive BT-474 breast cancer cell line.\textsuperscript{54} Regardless of the number of chelates per antibody, all six conjugates exhibited immunoreactive fractions greater than 0.93 (\(n = 3\) for each radiolabeled antibody). Blocking experiments performed with the addition of a vast excess (>500-fold) of unlabeled trastuzumab showed virtually no radioactive antibody binding and thus demonstrated the specificity of the \(^{64}\text{Cu}\)-DOTA- and \(^{89}\text{Zr}\)-DFO-T/N-trastuzumab. To assess the stability of radiolabeled bioconjugates, the \(^{64}\text{Cu}\)-DOTA- and \(^{89}\text{Zr}\)-DFO-T/N-trastuzumab formulations were incubated in human serum for 48 h and 7 d, respectively. Radio-TLC with an eluent of 50 mM EDTA (pH 5.0) illustrated that both sets of conjugates were >96% stable after the incubation period in all cases (Table 1).

### Table 2. Biodistribution Data of \(^{64}\text{Cu}\)-DOTA-T/N-trastuzumab versus Time in Mice Bearing Bilateral s.c. BT-474 (HER2-positive) and MDA-MB-468 (HER2-negative) Xenografts (\(n = 4\) for Each Time Point)

| Time (h) | Blood | HER2+ Tumor | HER2- Tumor | Heart | Lungs | Liver | Spleen | Stomach | Intestine | Kidney | Muscle | Bone |
|---------|-------|-------------|-------------|-------|-------|-------|-------|---------|----------|-------|--------|------|
| 6       | 19.2±5.2 | 10.4±4.6 | 6.2±1.1 | 4.9±1.1 | 12.2±1.2 | 11.5±2.0 | 11.1±5.5 | 1.9±0.7 | 3.5±1.5 | 5.5±0.9 | 0.6±0.2 | 3.1±2.7 |
| 12      | 16.2±4.1 | 23.9±4.6 | 8.6±2.7 | 6.7±2.7 | 9.5±1.5 | 10.1±1.4 | 1.0±1.3 | 2.1±0.3 | 2.1±0.3 | 4.9±1.0 | 0.7±0.4 | 1.4±0.1 |
| 24      | 10.5±3.6 | 26.1±4.8 | 9.0±0.9 | 4.3±0.8 | 6.4±2.3 | 9.7±1.3 | 1.7±0.1 | 1.7±0.1 | 2.3±0.3 | 2.9±1.6 | 0.6±0.3 | 1.4±0.3 |
| 48      | 11.2±1.6 | 44.0±7.7 | 8.7±2.5 | 4.3±1.2 | 8.0±1.3 | 6.4±0.2 | 1.3±0.3 | 2.4±0.3 | 3.1±1.7 | 5.7±0.4 | 1.0±0.2 | 1.1±0.2 |
| 72      | 11.8±1.3 | 55.1±2.3 | 11.7±1.3 | 5.7±3.1 | 9.5±0.4 | 8.1±0.9 | 1.8±0.2 | 2.0±0.4 | 4.5±0.4 | 6.9±1.4 | 0.8±0.1 | 2.6±1.1 |

In the \(^{64}\text{Cu}\)-DOTA-T/N-trastuzumab biodistribution experiment (Table 2), high specific uptake is observed in the HER2-positive BT-474 tumor, with the %ID/g increasing from 10.4 ± 4.6 at 6 h to 55.1 ± 2.3 at 72 h (tumor/muscle ratios of 17.3 ± 7.1 and 68.8 ± 8.0, respectively). By comparison, far lower levels of \(^{64}\text{Cu}\)-DOTA-T/N-trastuzumab uptake were seen in the HER2-negative MDA-MB-468 tumors. As expected, over the course of the experiment a concomitant decrease in the %ID/g in the blood (from 19.2 ± 5.2 at 6 h to 11.8 ± 1.3 at 72 h) also occurred. The organs with the highest background uptake were the lungs, liver, and spleen; though the uptake in these organs was at its highest point at 6 h, and by 72 h, the tumor/organ ratios for each of these organs were 5.8 ± 0.3, 6.8 ± 0.8, and 8.0 ± 1.6, respectively (see Supporting Information for complete table of tumor/organ ratios). Low levels of uptake were observed in the heart, stomach, small intestine, large intestine, kidney, muscle, and bone. Taken together, these results plainly indicate that \(^{64}\text{Cu}\)-DOTA-T/N-trastuzumab is an effective imaging agent for the delineation of the HER2-positive BT-474 xenografts. Perhaps just as importantly, these results are consistent with those previously reported for \(^{64}\text{Cu}\)-DOTA-T/N-trastuzumab conjugates, though the literature investigation used HER2-positive and HER2-negative non-small cell lung cancer cell lines.\textsuperscript{65} Interestingly, far lower background liver uptake was observed in our study, and while comparisons between different tumor models systems may bear some risks, this discrepancy suggests a lower rate of \(^{64}\text{Cu}\) decomplexation in our system.

Similarly positive results were observed in the \(^{89}\text{Zr}\)-DFO-T/N-trastuzumab biodistribution experiments (Table 3). Initially very high blood activity levels decreased over the course of the experiment, from 42.2 ± 8.8%ID/g at 6 h to 18.2 ± 3.3%ID/g at 120 h. More importantly, high specific uptake was observed in the HER2-positive BT474 tumors, peaking at over 75%ID/g at 72 h postinjection (tumor to muscle ratio: 34.1 ± 12.7). In contrast, the uptake in the HER2-negative MDA-MB-468 tumors was significantly lower, starting at 11.2 ± 5.8%ID/g at 6 h and peaking at 120 h at 17.6 ± 3.9%ID/g. Highest background uptake was observed in the lungs, liver, spleen, and kidney, with uptake values ranging from 7 to 17%ID/g and typically decreasing over the course of the experiment. Maximum tumor to organ ratio
The bone uptake of $^{89}$Zr has been reported on a number of occasions.

| 6 h  | 24 h  | 48 h  | 72 h  | 96 h  | 120 h |
|------|-------|-------|-------|-------|-------|
| blood| 42.2 ± 8.8 | 37.4 ± 3.4 | 24.1 ± 6.1 | 24.1 ± 8.2 | 20.2 ± 2.1 | 18.2 ± 3.3 |
| HER2+ tumor| 22.9 ± 6.6 | 48.6 ± 6.9 | 64.0 ± 6.8 | 75.1 ± 7.6 | 72.2 ± 7.9 | 69.8 ± 3.9 |
| HER2- tumor| 11.2 ± 5.0 | 13.4 ± 4.1 | 14.5 ± 8.8 | 16.8 ± 2.7 | 16.7 ± 6.8 | 17.6 ± 1.7 |
| heart| 24.1 ± 5.7 | 17.7 ± 6.9 | 6.0 ± 3.2 | 9.6 ± 3.8 | 8.5 ± 3.2 | 10.4 ± 0.9 |
| lungs| 15.5 ± 2.4 | 15.8 ± 5.4 | 10.5 ± 4.6 | 11.9 ± 4.8 | 13.8 ± 8.1 | 12.9 ± 3.2 |
| liver| 24.2 ± 5.0 | 17.6 ± 4.7 | 16.0 ± 6.5 | 15.8 ± 1.4 | 12.8 ± 2.7 | 13.5 ± 7.3 |
| spleen| 11.3 ± 1.2 | 14.2 ± 5.3 | 15.0 ± 5.1 | 14.9 ± 5.2 | 12.6 ± 6.5 | 10.8 ± 5.3 |
| stomach| 6.7 ± 1.4 | 3.1 ± 0.2 | 2.5 ± 0.7 | 2.0 ± 0.5 | 1.9 ± 0.4 | 1.8 ± 0.3 |
| small intestine| 8.4 ± 1.7 | 6.3 ± 0.3 | 7.9 ± 2.9 | 4.8 ± 1.1 | 3.8 ± 0.9 | 4.4 ± 0.8 |
| large intestine| 4.5 ± 1.0 | 2.1 ± 0.6 | 1.7 ± 0.2 | 2.3 ± 0.9 | 1.0 ± 0.3 | 1.4 ± 0.5 |
| kidney| 15.9 ± 5.4 | 13.3 ± 1.2 | 7.0 ± 1.3 | 11.6 ± 2.6 | 10.2 ± 0.3 | 8.7 ± 2.9 |
| muscle| 2.2 ± 0.6 | 2.3 ± 0.2 | 1.6 ± 0.5 | 2.2 ± 0.4 | 2.2 ± 0.2 | 2.5 ± 0.2 |
| bone| 12.1 ± 0.8 | 13.2 ± 2.4 | 14.6 ± 3.8 | 15.2 ± 1.9 | 14.7 ± 3.1 | 15.1 ± 1.5 |

The initial blood levels are higher in the $^{89}$Zr-DFO-T/N-trastuzumab biodistribution than at the corresponding time points in $^{64}$Cu-DOTA-T/N-trastuzumab. The exception to this is the HER2-positive BT-474 tumor for $^{89}$Zr-DFO-T/N-trastuzumab, where the uptake value (11.1% ID/g) is remarkably similar to the $^{64}$Cu-DOTA-T/N-trastuzumab (11.2% ID/g). The biodistribution data obtained with $^{89}$Zr-DFO-T/N-trastuzumab or $^{89}$Zr-DFO is, of course, out of the scope of this work, but it is interesting to note that the bone uptake does not increase dramatically over the course of the experiment, suggesting that the majority of $^{89}$Zr deposition in the bone occurs very soon after injection.

Overall, these results are generally consistent with those previously reported for $^{89}$Zr-trastuzumab biodistribution by Munnink et al. and Holland et al. No other data have been published on the uptake of $^{89}$Zr-trastuzumab in HER2-negative MDA-MB-468 tumors; however, the uptake value obtained at 24 h in this study (13.4 ± 4.1% ID/g) is remarkably similar to the value obtained at the same time point in the blocking experiment (200 µg additional unlabeled trastuzumab) performed by Holland et al. with BT-474 cells: 13.5 ± 4.8% ID/g. While this latter experiment is not, of course, directly comparable, it does help establish a baseline for the nonspecific tumor uptake of radiolabeled antibody.

Overall, two key differences are evident upon comparing the biodistribution data obtained with $^{89}$Zr-DFO-T/N-trastuzumab and $^{64}$Cu-DOTA-T/N-trastuzumab. The first, increased bone uptake in the $^{89}$Zr-DFO-T/N-trastuzumab experiment, is easily explained: free $^{89}$Zr$^{4+}$ is a bone-seeking radiometal, while free $^{64}$Cu$^{2+}$ has not been shown to accumulate in bone. Second, the HER2-specific tumor uptake and background signal (including initial blood levels) are higher in the $^{89}$Zr-DFO-T/N-trastuzumab biodistribution than at the corresponding time points in the $^{64}$Cu-DOTA-T/N-trastuzumab experiment. For example, at 6 h, the blood levels for $^{89}$Zr-DFO-T/N-trastuzumab are 42.2 ± 8.8% ID/g, while they are 19.2 ± 5.2% ID/g for $^{64}$Cu-DOTA-T/N-trastuzumab. Later, at 48 h, the uptake in the HER2-positive BT-474 tumor for $^{89}$Zr-DFO-T/N-trastuzumab is 72.2 ± 7.9% ID/g, while for $^{64}$Cu-DOTA-T/N-trastuzumab, it is 44.0 ± 7.7% ID/g. Moreover, at 48 h, the liver uptake of $^{89}$Zr-DFO-T/N-trastuzumab stands at 12.8 ± 2.7% ID/g, while it is 6.4 ± 0.2% ID/g for $^{64}$Cu-DOTA-T/N-trastuzumab at the same time point. It is possible that these variations in background uptake result from differences in the metabolism of the $^{89}$Zr-DFO- and $^{64}$Cu-DOTA-modified antibodies. The increase in HER2-specific uptake of the $^{89}$Zr-DFO-T/N-trastuzumab is somewhat more puzzling, though these data are consistent with that obtained in other investigations of $^{64}$Cu- and $^{89}$Zr-trastuzumab.

In the case of small peptides, it has been previously reported that the identity of the radiometal may play a role in the uptake of antibodies. The increase in HER2+ tumor uptake and background signal (including blood, spleen, liver, and muscle) is remarkably similar in the two experiments. Despite these differences, the biodistribution data plainly illustrate that both radiolabeled constructs are selectively and significantly taken up in the HER2-positive tumors. In addition, and perhaps more important in light of the goals of the investigation, the overall trends observed in uptake and tumor/organ ratio are strikingly similar in the two experiments.

**Small Animal PET Imaging.** Small animal PET imaging experiments were performed in order to further evaluate the in vivo behavior of the two radiometalated biocoujungates. In each case, nude mice (n = 5 for each construct) bearing bilateral BT-474 (HER2-positive) and MDA-MB-468 (HER2-negative) xenografts were injected via tail vein with either $^{64}$Cu-DOTA-T/N-trastuzumab (11.1–12.9 MBq [300–345 µCi]) or $^{89}$Zr-DFO-T/N-trastuzumab (10.7–11.8 MBq [290–320 µCi]). The animals were subsequently imaged periodically from injection (t = 0 h) to 48 h ($^{64}$Cu) or 120 h ($^{89}$Zr). The results clearly indicate that both constructs are taken up significantly and selectively in the HER2-positive BT-474 tumors (shown in Figures 3 and 4). In the case of $^{64}$Cu-DOTA-T/N-trastuzumab, high blood pool activity and some background uptake are evident at the early time points, but over the course of the experiment, the
signal in the BT-474 tumor increases significantly to a point at which it is easily the most intense feature in the PET image. Similarly, for $^{89}$Zr-DFO-T/N-trastuzumab, some blood pool activity is evident at the earliest time point, but the tumor uptake increases steadily in the subsequent time points along with a concomitant decrease in any background activity. The images produced by the two conjugates are very similar, a result that is consistent with the sum of the data collected in this investigation. In each case, very little background uptake is evident in either the HER2-negative MDA-MB-468 tumor or other organs. The only significant differences, as in the biodistribution experiments, are enhanced tumor and background uptake in the $^{89}$Zr-DFO-T/N-trastuzumab images compared to those from $^{64}$Cu-DOTA-T/N-trastuzumab and slight bone uptake of the former construct. The bone uptake is not evident in the images displayed in Figure 5 but can be spotted (though faint) in a maximum intensity projection (see Supporting Information). Just as important as the imaging similarities between the two constructs in this study, the images obtained here are consistent with those reported for other $^{89}$Zr- and $^{64}$Cu-trastuzumab radioagents in the literature.

Radiolabeling Trastuzumab with a Two-Step Ligation Strategy. The modular strategy described to this point comprises three simple steps: norbornene modification, tetrazine-chelator ligation, and radiometalation. However, the versatility of the tetrazine-norbornene ligation makes an alternate route possible as well: a two-step procedure in which a norbornene-modified antibody is reacted with a radiometalated, chelator-modified tetrazine (Figure 5). Indeed, similar ligations of dienophiles with radiolabeled tetrazines have already been employed with success with $^{18}$F and $^{111}$In, though in these cases, a transcyclooctene dienophile was employed rather than a norbornene. 29–31 To demonstrate the feasibility of such a strategy with PET radiometals, Tz-DOTA (5 nmol) was radiolabeled with $^{64}$Cu (1.1–1.5 mCi) in 50 mM NH$_4$OAc pH 5.5 via incubation at 85°C for 1 h ($n = 3$ trials). After the 1 h incubation, the labeling reaction was purified via radio-HPLC, and the product was obtained in an uncorrected
radiochemical yield of 80 ± 3% with greater than 99% radiochemical purity and a specific activity of 160 ± 5 mCi/μmol. Subsequently, this 64Cu-Tz-DOTA was incubated with norbornene-modified trastuzumab (0.4 mg, 1.3 nmol, initial norbornene/mAb stoichiometry of 5:1) in PBS pH 7.4 (200 μL) at 37 °C. The progress of the reaction was monitored with radio-TLC, and after 3 h, the reaction was gauged to have reached completion. After purification via centrifugal filtration, the completed 64Cu-DOTA-T/N-trastuzumab conjugate was isolated in ~75% radiochemical purity with a specific activity of 1.0 ± 0.4 mCi/μg. Granted, this specific activity is somewhat lower than that obtained with the three-step method; however, further optimization, though outside of the scope of the work at hand, could no doubt raise this specific activity to levels on par with that achieved with the three-step strategy.

Ultimately, it is our belief that the three-step method is preferable as a modular strategy for radiolabeling antibodies. This method holds the key advantage of only involving a single and relatively rapid radiochemical step, thereby minimizing the amount of radiochemistry needed for the creation of the bioconjugates while simultaneously maximizing specific activities. However, it is clear from the work currently in the literature—particularly that of Devaraj et al. and Rossin et al.—that the two-step method holds significant potential as a strategy for pretargeted antibody or peptide imaging.23,24,27–29,31 In this application, a dienophile-modified biomolecule is first injected into a tumor-bearing animal and is permitted time to achieve its optimal biodistribution. Subsequently, a fluorophore- or radionuclide-modified tetrzine moiety is injected into the same animal and, due to the bioorthogonal nature of the tetrzine-dienophile ligation, could selectively react with the dienophile-modified biomolecule, resulting in specific localization of the marker. Indeed, both the optical and nuclear pretargeting strategies have shown very promising results. It is important to note, though, that the pretargeting systems described in the literature employ more reactive, less stable trans-cyclooctene dienophiles instead of the more stable, less reactive norbornene dienophile used in this study. Experiments are currently underway toward the creation of a pretargeting system for positron-emitting radiometals employing more reactive dienophiles.

**CONCLUSION**

In summary, herein we report the development of a modular system for the radiometalation of antibodies using the inverse electron demand Diels–Alder cycloaddition between tetrzine and norbornene. The strategy involves three facile, rapid, and biocompatible steps: modification of an antibody with norbornene, ligation of a chelator-modified tetrzine, and radiometalation. In this proof of concept investigation, the methodology was employed to create bioconjugates of the HER2-specific antibody trastuzumab bearing the positron-emitting radiometals 64Cu and 89Zr in high radiochemical purity and specific activity. For a given initial loading of norbornene, the DOTA- and DFO-modified constructs were shown to have identical numbers of chelates per antibody, and all of the radiolabeled 64Cu-DOTA- and 89Zr-DFO-bioconjugates displayed high serum stability and immuno-reactivity. Finally, both radiolabeled bioconjugates were used in in vivo biodistribution and PET imaging studies with mice bearing HER2-positive (BT-474) and HER2-negative (MDA-MB-468) breast cancer xenografts. Both antibody constructs were shown to have significant and specific uptake in the HER2-positive tumor with low uptake in the HER2-negative tumor and other tissues.

This strategy does not necessarily offer a significant improvement in facility compared to popular DOTA-NHS or DFO-NCS antibody modification protocols; more importantly, however, it creates a modular platform in which a common, covalently modified antibody can be modified with a wide variety of chelators and radiometals. Given that different radiometals often require different chelators—and thus the use and optimization of different modification pathways—this methodology could no doubt aid in the rapid and robust construction of diverse radio-pharmaceuticals from a single antibody stock. Further, this modular system could facilitate the creation of meaningful comparisons between bioconjugates labeled with different radiometals: as we have shown, because the chelator-modified antibodies are synthesized using identical ligation conditions, the immunoreactivity and chelator/antibody ratios of the resultant bioconjugates are likewise nearly identical regardless of the identity of the tetrzine—chelator pair.

Ultimately, therefore, this modular methodology has the potential not only to significantly aid in the synthesis and development of new radiometalated bioconjugates for PET, SPECT, and radiotherapy, but also to advance cross-pollination and constructive comparisons between radiopharmaceuticals employing diverse metallic radionuclides.
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