



Chemoenzymatic Strategy for the Synthesis of Site-Specifically Labeled Immunoconjugates for Multimodal PET and Optical Imaging

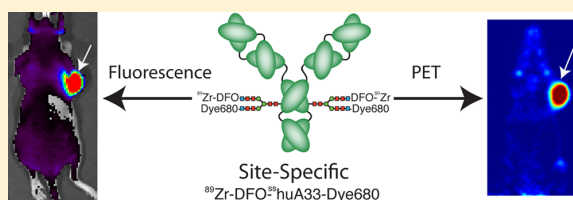
Brian M. Zeglis,[†] Charles B. Davis,[†] Dalya Abdel-Atti,[†] Sean D. Carlin,[†] Aimei Chen,[‡] Robert Aggeler,[‡] Brian J. Agnew,[‡] and Jason S. Lewis^{*,†}

[†]Department of Radiology, Memorial Sloan Kettering Cancer Center, New York, New York 10065, United States

[‡]Biosciences Division, Thermo Fisher Scientific, Eugene, Oregon 97402, United States

S Supporting Information

ABSTRACT: The complementary nature of positron emission tomography (PET) and optical imaging (OI) has fueled increasing interest in the development of multimodal PET/OI probes that can be employed during the diagnosis, staging, and surgical treatment of cancer. Due to their high selectivity and affinity, antibodies have emerged as promising platforms for the development of hybrid PET/OI agents. However, the lack of specificity of many bioconjugation reactions can threaten immunoreactivity and lead to poorly defined constructs. To circumvent this issue, we have developed a chemoenzymatic strategy for the construction of multimodal PET/OI immunoconjugates that have been site-specifically labeled on the heavy chain glycans. The methodology consists of four steps: (1) the enzymatic removal of the terminal galactose residues on the heavy chain glycans; (2) the enzymatic incorporation of azide-bearing galactose (GalNAz) residues into the heavy chain glycans; (3) the strain-promoted click conjugation of chelator- and fluorophore-modified dibenzocyclooctynes to the azide-modified sugars; and (4) the radiolabeling of the immunoconjugate. For proof-of-concept, a model system was created using the colorectal cancer-targeting antibody huA33, the chelator desferrioxamine (DFO), the positron-emitting radiometal ⁸⁹Zr, and the near-infrared fluorescent dye Alexa Fluor 680. The bioconjugation strategy is robust and reproducible, reliably producing well-defined and immunoreactive conjugates labeled with ⁸⁹Zr, Alexa Fluor 680, or an easily and precisely tuned mixture of the two reporters. In *in vivo* PET and fluorescence imaging experiments, a hybrid ⁸⁹Zr- and Alexa Fluor 680-labeled huA33 conjugate displayed high levels of specific uptake (>45% ID/g) in athymic nude mice bearing A33 antigen-expressing SW1222 colorectal cancer xenografts.



INTRODUCTION

Over the past 30 years, molecular imaging has transformed cancer care. The ability to noninvasively acquire anatomical and functional information about tumors has aided clinicians in all stages of cancer management, from diagnosis to staging to treatment.^{1–3} While the vast majority of imaging agents are specific to a single modality, recent years have played witness to a surge in the development of multimodal probes.^{4–7} In this regard, the complementary nature of positron emission tomography (PET) and optical imaging (OI) have made hybrid PET/OI probes particularly promising tools. While PET radiopharmaceuticals enable noninvasive whole body imaging and provide functional and anatomical information about lesions, near-infrared fluorescence (NIRF) optical imaging probes facilitate the high resolution imaging of tumor margins during surgical resection.^{3,5,7,8}

Given their exquisite selectivity and affinity for their molecular targets, antibodies have emerged as particularly exciting platforms for the development of hybrid PET/OI agents. In the past few years, preclinical investigations employing PET/OI immunoconjugates targeting HER2, CD20, VEGF, and CD105 have emerged in the literature.^{9–15} Yet, despite their promise, one critical obstacle to the

development of PET/OI immunoconjugates is the lack of site-specificity in the bioconjugation of radionuclides and fluorophores. At present, the vast majority of bioconjugation techniques rely on reactions between bifunctional probes and amino acids, most often lysines.¹² Antibodies possess varying numbers of lysine residues, and thus controlling the precise molecular location of conjugation reactions is impossible. This lack of site-specificity can impair immunoreactivity if the conjugation reaction inadvertently occurs in or around the antigen binding domain. Furthermore, random bioconjugation strategies yield inadequately chemically defined constructs and can complicate the reliable reproduction of results from one antibody to the next. Not surprisingly, the potential complications of random bioconjugation are magnified in dual-labeled PET/OI constructs, as two types of reporter moieties are being appended to the antibody in two separate conjugation reactions.

In order to circumvent these issues, considerable effort has been dedicated to the development of strategies for the site-

Received: October 31, 2014

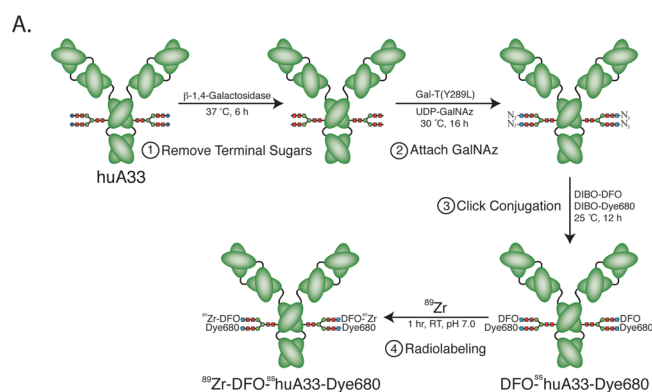
Revised: November 21, 2014

Published: November 24, 2014

specific conjugation of payloads to antibodies.^{16–24} While promising, many of these methodologies are limited by their use of expensive and complex work flows and/or result in poor reproducibility of labeling between different antibodies. For example, one emergent group of strategies for the site-specific modification of antibodies—and the creation of antibody–drug conjugates in particular—has relied on the reaction between bifunctional, maleimide-bearing constructs and free cysteines in antibodies.^{19,25,26} These methodologies have largely proven successful. However, this sulfhydryl-based chemistry requires either the reduction of existing, natural disulfide bonds within the antibody or the introduction of free cysteine residues via genetic engineering, processes which require significant optimization and limit the general applicability of these strategies.

As a more universal alternative, we have developed a chemoenzymatic method for the site-specific radiolabeling of antibodies.²⁷ This methodology leverages a promiscuous galactosyltransferase, an azide-modified substrate, and strain-promoted click chemistry to specifically modify the heavy chain glycans, the conserved, N-linked biantennary oligosaccharide chains positioned on the CH2 domain of the Fc region, far from the antigen binding regions.²⁸ The foundations of the approach lie in the glycoengineering work of Qasba, Hsieh-Wilson, and others, as well as the strain-promoted click chemistry work of Bertozzi and Boons.^{29–35} Admittedly, bioconjugation approaches based on the chemical manipulation of the heavy chain glycans predate our strategy.^{36,37} However, these earlier methods often require the prolonged exposure of the antibody to harsh redox conditions and have inherently high variability of labeling between different antibodies. It is also important to note that a number of descriptions of glycan-based bioconjugation strategies have appeared in the literature since our initial report; however, to the best of our knowledge, none of these have been applied to the development of multimodal probes.^{20–22,24}

Here, we report the development of site-specifically labeled immunoconjugates for the multimodal PET/OI imaging of colorectal cancer. The bioconjugation strategy has four steps: (1) the enzymatic removal of the terminal galactose residues on the heavy chain glycans with β -1,4-galactosidase; (2) the enzymatic incorporation of azide-bearing galactose (GalNAz) residues into the glycans using a substrate-permissive galactosyltransferase, Gal-T(Y289L); (3) the strain-promoted click conjugation of chelator- and fluorophore-modified dibenzocyclooctynes (DIBO) to the azide-modified sugars; and (4) the radiolabeling of the immunoconjugate (Figure 1). For proof-of-concept, a model system was created using the colorectal cancer-targeting antibody huA33, the positron-emitting radiometal ⁸⁹Zr, and the near-infrared fluorophore Alexa Fluor 680 (Dye680). The bioconjugation strategy proved robust, creating huA33 immunoconjugates with an easily tunable ratio of reporter moieties. Further, the methodology yielded a site-specifically labeled, hybrid PET/OI probe—⁸⁹Zr-DFO-⁸⁸huA33-Dye680—with high immunoreactivity *in vitro* and high efficacy *in vivo* in a murine model of colorectal cancer. Ultimately, one of the greatest strengths of this strategy is its modularity, as the enzymatic tagging of the glycans with GalNAz can be performed with any IgG antibody, and dibenzocyclooctynes can be conjugated easily to a wide variety of reporter molecules.



B.

Construct	DIBO-DFO:DIBO-Dye680 Ratio	DFO/mAb	Dye680/mAb	Total DOL
⁸⁸ huA33-DFO	100% DIBO-DFO	3.4 +/- 0.2	—	3.4 +/- 0.2
DFO- ⁸⁸ huA33-Dye680	3:1	2.9 +/- 0.1	0.5 +/- 0.1	3.4 +/- 0.2
DFO- ⁸⁸ huA33-Dye680	1:1	2.0 +/- 0.1	1.0 +/- 0.1	3.0 +/- 0.2
DFO- ⁸⁸ huA33-Dye680	1:3	1.0 +/- 0.1	1.3 +/- 0.2	2.3 +/- 0.3
⁸⁸ huA33-Dye680	100% DIBO-Dye680	—	1.6 +/- 0.2	1.6 +/- 0.2

Figure 1. (A) Scheme of the site-specific methodology for the chemoenzymatic synthesis of multimodal imaging immunoconjugates. (B) Some properties of the site-specifically labeled huA33 immunoconjugates discussed in this work. DOL = degree of labeling [(DFO + Dye680)/mAb]. Alexa Fluor 680 is represented as Dye680 in the figure.

RESULTS AND DISCUSSION

Design of the model system was the first step of the investigation. The huA33 antibody targets the A33 antigen, a transmembrane glycoprotein expressed on >95% of all colorectal cancers, and has shown considerable promise in the clinic as a platform for radioimmunoconjugates.³⁸ The ability to image colorectal cancer—particularly colorectal cancer metastatic to the liver—with PET during diagnosis and staging and with near-infrared fluorescence during surgical resection would certainly aid clinicians in the management of the disease. The positron-emitting radiometal ⁸⁹Zr was chosen due to the advantageous match between the radiometal's physical half-life ($t_{1/2} \sim 3.27$ d) and the multiday biological half-life of the antibody.³⁹ The selection of ⁸⁹Zr in turn mandates the choice of its octadentate, siderophore-derived chelator desferrioxamine (DFO).⁴⁰ Alexa Fluor 680 (Dye680) was chosen because of its near-infrared, tissue-penetrating 702 nm emission.

The site-specific modification process begins with the enzymatic manipulation of the heavy chain glycans. The antibody was first incubated with β -1,4-galactosidase at 37 °C for 6 h to expose the terminal GlcNAc sugars of the glycans. Subsequently, UDP-GalNAz and the promiscuous galactosyltransferase, Gal-T(Y289L), were added to the antibody solution, and the mixture was incubated at 30 °C for 16 h to append the azide-bearing galactose residues to the sugar chains. Since our initial report, these steps have been shortened from two 16 h incubations with an intermediate purification to a facile two enzyme, one pot reaction.²⁷ Next, the DFO-, Alexa Fluor 680-, and dual-labeled conjugates were synthesized. While the singly labeled constructs were created by incubating the site-specifically labeled huA33-N₃ construct (⁸⁸huA33-N₃) with DIBO-DFO or DIBO-Alexa Fluor 680 (DIBO-Dye680), the dual labeled constructs were synthesized via incubation with 1:3, 1:1, or 3:1 mixtures of the two DIBOs. All of the immunoconjugates were purified using size exclusion

chromatography, and a cumulative yield of $84.8 \pm 8.4\%$ was obtained over the course of the three step procedure ($n = 20$). Isotopic dilution experiments reveal that ^{89}Zr -DFO had a degree of labeling of 3.4 ± 0.2 DFO/mAb, consistent with the near-quantitative labeling of four theoretically available terminal GlcNAc residues. In contrast, the degree of labeling of ^{89}Zr -Dye680 was 1.6 ± 0.1 Dye680/mAb. Admittedly, this is below the typical degree of labeling previously observed with other chelators and dyes ($\sim 3\text{--}3.5/\text{mAb}$) and may be a result of the size and/or hydrophobicity of the fluorophore, though efforts to fully understand the cause are ongoing.²⁷ For the hybrid probes, the ratio of conjugated DFO to Dye680 could be easily controlled by altering the initial reaction ratio of DIBO–DFO to DIBO–Dye680. For example, the degree of labeling of Dye680 increases from 0.5 ± 0.1 to 1.0 ± 0.1 to 1.3 ± 0.1 as the proportion of DIBO–Dye680 in the original reaction mixture is increased. Interestingly, the total degree of labeling ($[\text{DFO} + \text{Dye680}]/\text{mAb}$) decreases with increasing proportion of DIBO–Dye680 in the original reaction mixture, perhaps a result of the same phenomenon that leads to a lower degree of fluorescence labeling in the ^{89}Zr -huA33-Dye680 construct.

SDS-PAGE electrophoresis experiments were run in order to characterize and confirm the site-specificity of the bioconjugation approach. In the resulting gels, a distinct increase in the molecular weight of the heavy chain of the DFO-, Dye680-, and dual-labeled ^{89}Zr -huA33 constructs can be observed compared to unmodified huA33 and ^{89}Zr -huA33- N_3 (Figure 2). There is no

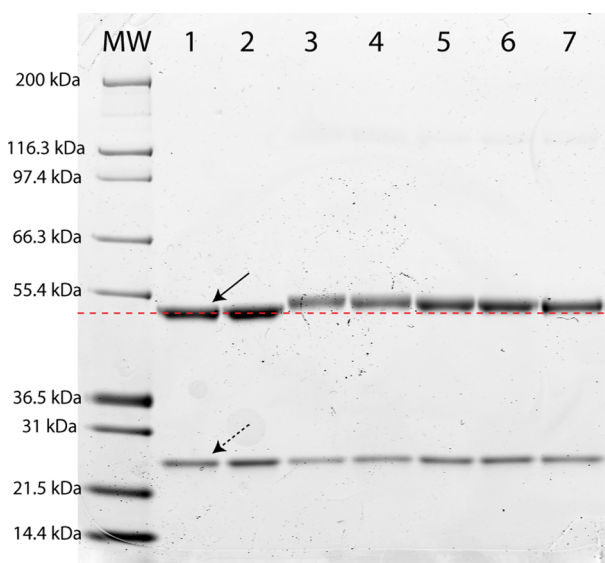


Figure 2. SDS-PAGE analysis of site-specifically labeled immunoconjugates. Lane 1: huA33. Lane 2: ^{89}Zr -huA33- N_3 . Lane 3: ^{89}Zr -huA33-DFO. Lanes 4, 5, and 6: DFO- ^{89}Zr -huA33-Dye680 synthesized with 3:1, 1:1, and 1:3 mixtures of DIBO-DFO:DIBO-Dye680, respectively. Lane 7: ^{89}Zr -huA33-Dye680. The solid and dotted arrows indicate the antibody heavy chain and light chain, respectively, and the red dotted line indicates the molecular weight of the unmodified heavy chain of huA33. MW = molecular weight ladder.

corresponding shift in the molecular weight of the light chain, strongly suggesting site-specific labeling. In additional experiments, the immunoconjugates were treated with PNGaseF, an amidase that specifically cleaves between the innermost sugar of the glycans and the asparagine residues of the antibody (see Supporting Information). Upon PNGaseF treatment, a drop in the molecular weight of the heavy chain is apparent for all of

the constructs, and, critically, the resulting bands are all shifted to the same molecular weight.

Ultimately, the DFO- ^{89}Zr -huA33-Dye680 construct bearing 1.0 ± 0.1 Dye680/mAb and 2.0 ± 0.2 DFO/mAb was chosen for further *in vitro* and *in vivo* study. For the sake of comparison, a dual-labeled DFO- ^{89}Zr -huA33-Dye680 construct bearing 1.0 ± 0.2 Dye680/mAb and 2.5 ± 0.4 DFO/mAb was also synthesized using a traditional, non-site-specific isothiocyanate-based coupling strategy (Figure 3A). Admittedly, the traditional

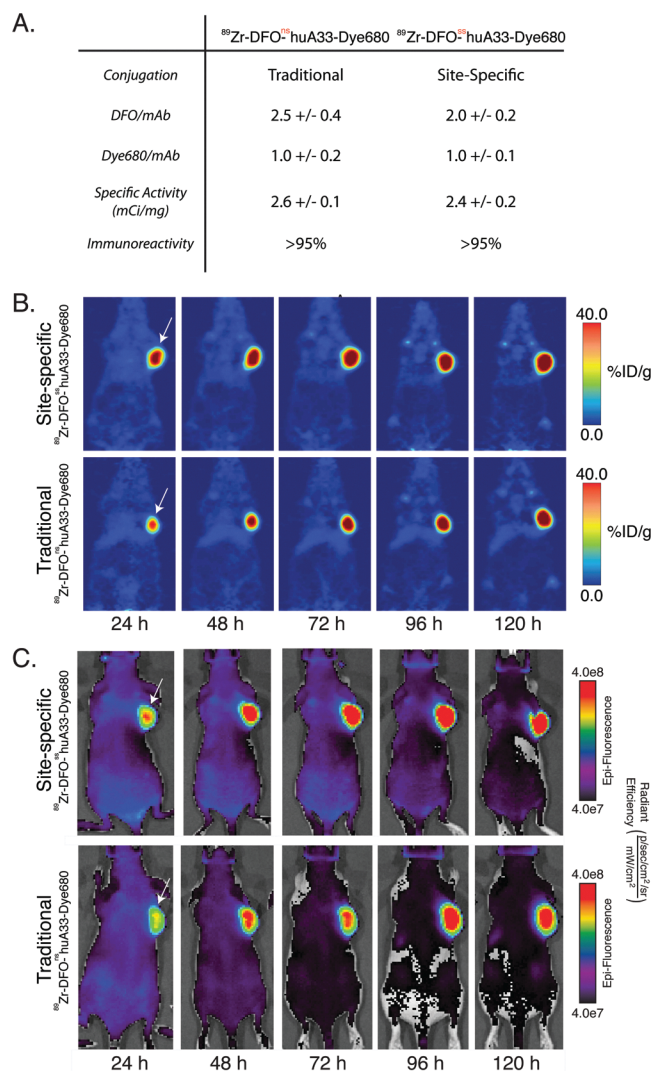


Figure 3. (A) Properties of site-specifically and traditionally labeled hybrid PET/OI immunoconjugates. PET (B) and fluorescence (C) images for ^{89}Zr -DFO- ^{89}Zr -huA33-Dye680 (top) and ^{89}Zr -DFO- ^{89}Zr -huA33-Dye680 (bottom) in mice bearing subcutaneous SW1222 xenografts. Mice were administered ^{89}Zr -DFO- ^{89}Zr -huA33-Dye680 or ^{89}Zr -DFO- ^{89}Zr -huA33-Dye680 [$180\text{--}200$ μCi ($72\text{--}80$ μg) in 200 μL 0.9% sterile saline] via lateral tail vein injection ($t = 0$) and imaged 24, 48, 72, 96, and 120 h postinjection. White arrows delineate the xenografts.

bioconjugation method is more rapid (total time = 2 h) and results in slightly higher yields ($89.9 \pm 4.9\%$); however, the cumbersome optimization required to obtain a traditionally conjugated construct with degrees of labeling analogous to DFO- ^{89}Zr -huA33-Dye680 underscores the robustness and reproducibility of the chemoenzymatic approach.

Both DFO-^{ss}huA33-Dye680 and DFO-^{ns}huA33-Dye680 (1000 μ g) were subsequently labeled with ⁸⁹Zr via incubation with ⁸⁹Zr (3.0–3.2 mCi) in PBS buffer at pH 7.0–7.5 for 1 h at room temperature. After purification via size exclusion chromatography, both probes were isolated in >99% radiochemical purity. The specific activities of the site-specific and traditionally synthesized constructs were similar, 2.4 ± 0.2 mCi/mg and 2.6 ± 0.1 mCi/mg, respectively. Both DFO-^{ss}huA33-Dye680 (0.95 ± 0.04) and DFO-^{ns}huA33-Dye680 (0.93 ± 0.06) also displayed high immunoreactive fractions when assayed using A33 antigen-expressing SW1222 colorectal cancer cells. While a more significant increase in immunoreactivity might be expected for the site-specific methodology, huA33 is a well-optimized and robust antibody. We expect the immunoreactivity benefits of this approach to be more evident on immunoglobulins that are known to lose a significant amount of activity upon conjugation using standard conjugation chemistries. Finally, to assay the serum stability of the constructs, both ⁸⁹Zr-DFO-^{ss}huA33-Dye680 and ⁸⁹Zr-DFO-^{ns}huA33-Dye680 were incubated in human serum for 7 d at 37 °C. After 7 days, radioTLC analysis revealed both constructs to be >95% intact.

In order to investigate the *in vivo* behavior of the multimodal constructs, small animal PET and fluorescence imaging experiments were performed in a mouse model of colorectal cancer (Figure 3B,C). To this end, nude mice bearing subcutaneous A33 antigen-expressing SW1222 xenografts were intravenously injected with either ⁸⁹Zr-DFO-^{ss}huA33-Dye680 or ⁸⁹Zr-DFO-^{ns}huA33-Dye680 (180–200 μ Ci, 72–80 μ g) and imaged with both PET and fluorescence at 24, 48, 72, 96, and 120 h postinjection. Both PET and fluorescence images clearly show that both immunoconjugates accumulate significantly in the tumors. At 24 h postinjection, uptake in the tumor is pronounced, but the constructs are also clearly visible—particularly via PET—in the blood and heart, reducing tumor-to-background contrast. Over the course of the experiment, however, the immunoconjugates clear from the blood and accumulate in the xenografts such that the tumors are prominently delineated from nontarget tissue with excellent tumor-to-background contrast. Complementary acute biodistribution experiments confirmed the imaging results (see Supporting Information). For ⁸⁹Zr-DFO-^{ss}huA33-Dye680, tumoral uptake values of $45.9 \pm 7.9\%$ ID/g and $45.7 \pm 8.1\%$ ID/g were observed at 48 and 120 h, respectively, resulting in tumor-to-muscle activity ratios of 30.1 ± 8.2 and 33.5 ± 15.5 at the two time-points. Critically, in the same biodistribution experiment, a blocking experiment in which an excess of 300 μ g unlabeled DFO-^{ss}huA33-Dye680 was co-injected with the radioimmunoconjugate reduced tumoral uptake at 48 h from $45.7 \pm 13.1\%$ ID/g to $15.7 \pm 4.1\%$ ID/g, demonstrating antigen specificity. Finally, *ex vivo* fluorescence microscopy and autoradiography were performed on xenografts excised from mice injected with ⁸⁹Zr-DFO-^{ss}huA33-Dye680. The fluorescence and radioactivity are clearly colocalized in the same regions of the tumor, the expected results for a dual labeled imaging agent. Taken together, this investigation clearly demonstrates that the *in vitro* and *in vivo* behavior of ⁸⁹Zr-DFO-^{ss}huA33-Dye680 is comparable to that of the traditionally constructed agent.

CONCLUSION

We have developed a methodology for the construction of site-specifically labeled multimodal PET/OI immunoconjugates.

The synthetic strategy has proven to be robust and reliable, and we have used it to create a hybrid ⁸⁹Zr-DFO- and Alexa Fluor 680-labeled probe shown to be as effective *in vivo* in a murine model of colorectal cancer as an analogous construct synthesized using traditional non-site-specific methods. Importantly, the ratio of chelator to fluorophore can be easily tuned according to the needs of the experiment. If high specific activity is a priority, the ratio of chelator-to-fluorophore can be increased. Alternatively, if fluorescent brightness is the goal, the relative number of fluorophores can be increased. Perhaps even more critically, the overall strategy is completely modular, as the chemoenzymatic GalNAz conjugation can be employed with any antibody, and myriad reporters can be appended to dibenzocyclooctynes. Ultimately, we believe that this methodology could have a transformational impact on the way multimodal immunoconjugates are developed.

ASSOCIATED CONTENT

Supporting Information

Reagents and general procedures; detailed experimental methods on chemical syntheses, bioconjugation procedures, *in vitro*, and *in vivo* experiments; diagram of the strain-promoted azide-cyclooctyne click reaction; synthesis of DIBO-Alexa Fluor 680; SDS-PAGE analysis of PNGase-F treated immunoconjugates; tables of biodistribution data, both absolute uptake values and tumor-to-tissue activity ratios. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*E-mail: lewisj2@mskcc.org. Phone: 1-646-888-3039. Fax: 1-646-888-3059.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Services provided by the MSKCC Small-Animal Imaging Core Facility were supported in part by NIH Grants R24 CA83084 and P30 CA08748. The authors also thank the NIH (Award 1K99CA178205-01A1), the DoD (Award W81XWH-12-1-0029, B.M.Z.), Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research, and the Experimental Therapeutics Center of Memorial Sloan Kettering Cancer Center for their generous funding.

ABBREVIATIONS

DIBO, dibenzocyclooctyne; UDP, uridine-5'-diphosphate; GalNAz, *N*-azidoacetyl galactosamine; DFO, desferrioxamine; Dye680, Alexa Fluor 680; ss, site-specific; ns, non-site-specific

REFERENCES

- (1) Wu, A. M., and Olafsen, T. (2008) Antibodies for molecular imaging of cancer. *Cancer J.* 14, 191–197.
- (2) Kircher, M. F., Hricak, H., and Larson, S. M. (2012) Molecular imaging for personalized cancer care. *Mol. Oncol.* 6, 182–195.
- (3) Michalski, M. H., and Chen, X. (2011) Molecular imaging in cancer treatment. *Eur. J. Nucl. Med. Mol. Imag.* 38, 358–377.
- (4) Cherry, S. R. (2009) Multimodality imaging: beyond PET/CT and SPECT/CT. *Sem. Nucl. Med.* 39, 348–353.
- (5) Louie, A. Y. (2010) Multimodality imaging probes: Design and challenges. *Chem. Rev.* 110, 3146–3195.

- (6) Seibold, U., Wangler, B., Schirmacher, R., and Wangler, C. (2014) Bimodal imaging probes for combined PET and OI: Recent developments and future directions for hybrid agent development. *Biomed. Res. Int.*, 1–13.
- (7) te Velde, E. A., Veerman, T., Subramaniam, V., and Ruers, T. (2010) The use of fluorescent dyes and probes in surgical oncology. *Eur. J. Surg. Oncol.* 36, 6–15.
- (8) Frangioni, J. V. (2003) In vivo near-infrared fluorescence imaging. *Curr. Opin. Chem. Biol.* 7, 626–634.
- (9) Xu, H., Baidoo, K., Gunn, A. J., Boswell, C. A., Milenic, D. E., Choyke, P. L., and Brechbiel, M. W. (2007) Design, synthesis, and characterization of a dual modality positron emission tomography and fluorescence imaging agent for monoclonal antibody tumor-targeted imaging. *J. Med. Chem.* 50, 4759–4765.
- (10) Paudyal, P., Paudyal, B., Iida, Y., Oriuchi, N., Hanaoka, H., Tominaga, H., Ishikita, T., Yoshioka, H., Higuchi, T., and Endo, K. (2009) Dual functional molecular imaging probe targeting CD20 with PET and optical imaging. *Oncol. Rep.* 22, 115–119.
- (11) Sampath, L., Kwon, S., Ke, S., Wang, W., Schiff, R., Mawad, M. E., and Sevick-Muraca, E. M. (2007) Dual-labeled trastuzumab-based imaging agent for the detection of human epidermal growth factor receptor 2 overexpression in breast cancer. *J. Nucl. Med.* 48, 1501–1510.
- (12) Cohen, R., Vugts, D. J., Stigter-van Walsum, M., Visser, G. W. M., and van Dongen, G. (2013) Inert coupling of IRDye800CW and zirconium-89 to monoclonal antibodies for single- or dual-mode fluorescence and PET imaging. *Nat. Protoc.* 8, 1010–1018.
- (13) Hong, H., Zhang, Y., Severin, G. W., Yang, Y. N., Engle, J. W., Niu, G., Nickles, R. J., Chen, X. Y., Leigh, B. R., Barnhart, T. E., and Cai, W. B. (2012) Multimodality imaging of breast cancer experimental lung metastasis with bioluminescence and a monoclonal antibody dual-labeled with Zr-89 and IRDye 800CW. *Mol. Pharmaceutics* 9, 2339–2349.
- (14) Zhang, Y., Hong, H., Severin, G. W., Engle, J. W., Yang, Y. A., Goel, S., Nathanson, A. J., Liu, G., Nickles, R. J., Leigh, B. R., Barnhart, T. E., and Cai, W. B. (2005) ImmunoPET and near-infrared fluorescence imaging of CD105 expression using a monoclonal antibody dual-labeled with Zr-89 and IRDye 800CW. *Am. J. Transl. Res.* 4, 333–346.
- (15) Sampath, L., Kwon, S., Hall, M. A., Price, R. E., and Sevick-Muraca, E. M. (2010) Detection of cancer metastases with a dual-labeled near-infrared/positron emission tomography imaging agent. *Trans. Oncol.* 3, 307–U71.
- (16) Hussain, A. F., Kampmeier, F., von Felbert, V., Merk, H. F., Tur, M. K., and Barth, S. (2011) SNAP-Tag technology mediates site-specific conjugation of antibody fragments with a photosensitizer and improves target specific phototoxicity in tumor cells. *Bioconjugate Chem.* 22, 2487–2495.
- (17) Jeong, J. M., Lee, J., Paik, C. H., Kim, D. K., Lee, D. S., Chung, J. K., and Lee, M. C. (2004) Site-specific ^{99m}Tc-labeling of antibody using dihydrazinophthalazine (DHZ) conjugation to Fc region of heavy chain. *Arch. Pharm. Res.* 27, 961–967.
- (18) Kampmeier, F., Ribbert, M., Nachreiner, T., Dembski, S., Beaufils, F., Brecht, A., and Barth, S. (2009) Site-specific, covalent labeling of recombinant antibody fragments via fusion to an engineered version of 6-O-alkylguanine DNA alkyltransferase. *Bioconjugate Chem.* 20, 1010–1015.
- (19) Tinianow, J. N., Gill, H. S., Ogasawara, A., Flores, J. E., Vanderbilt, A. N., Luis, E., Vandlen, R., Darwish, M., Junutula, J. R., Williams, S. P., and Marik, J. (2010) Site-specifically Zr-89-labeled monoclonal antibodies for ImmunoPET. *Nucl. Med. Biol.* 37, 289–297.
- (20) Dennler, P., Chiotellis, A., Fischer, E., Bregeon, D., Belmant, C., Gauthier, L., Lhospice, F., Romagne, R., and Schibli, R. (2014) Transglutaminase-based chemo-enzymatic conjugation approach yields homogeneous antibody–drug conjugates. *Bioconjugate Chem.* 25, 569–578.
- (21) Zhou, Q., Stefano, J. E., Manning, C., Kyazike, J., Chen, B., Gianolio, D. A., Park, A., Busch, M., Bird, J., Zheng, X., Simonds-Mannes, H., Kim, J., Gregory, R. C., Miller, R. J., Brondyk, W. H., Dhal, P. K., and Pan, C. Q. (2014) Site-specific antibody–drug conjugation through glycoengineering. *Bioconjugate Chem.* 25, 510–520.
- (22) Okeley, N. M., Toki, B. E., Zhang, X., Jeffrey, S. C., Burke, P. J., Alley, S. C., and Senter, P. D. (2013) Metabolic engineering of monoclonal antibody carbohydrates for antibody–drug conjugation. *Bioconjugate Chem.* 24, 1650–1655.
- (23) Boswell, C. A., Marik, J., Elowson, M. J., Reyes, N. A., Ulufatu, S., Bumbaca, D., Yip, V., Mundo, E. E., Majidy, N., Van Hoy, M., Goriparthi, S. N., Trias, A., Gill, H. S., Williams, S. P., Junutula, J. R., Fielder, P. J., and Khawli, L. A. (2013) Enhanced tumor retention of a radiohalogen label for site-specific modification of antibodies. *J. Med. Chem.* 56, 9418–9426.
- (24) Li, Z., Fang, T., and Boons, G. J. (2014) Preparation of well-defined antibody–drug conjugates through glycan remodeling and strain-promoted azide–alkyne cycloadditions. *Angew. Chem., Int. Ed.* 53, 7179–7182.
- (25) Massa, S., Xavier, C., De Vos, J., Caveliers, V., Lahoutte, T., Muyldermans, S., and Devoogdt, N. (2014) Site-specific labeling of cysteine-tagged camelid single-domain antibody-fragments for use in molecular imaging. *Bioconjugate Chem.* 25, 979–988.
- (26) Pillow, T. H., Tien, J., Parsons-Repointe, K. L., Bhakta, S., Li, H., Staben, L. R., Li, G., Chuh, J., Fourie-O'Donohue, A., Darwish, M., Yip, V., Liu, L., Leipold, D. D., Su, D., Wu, E., Spencer, S. D., Shen, B.-Q., Xu, K., Kozak, K. R., Raab, H., Vandlen, R., Phillips, G. D. L., Scheller, R. H., Polakis, P., Sliwowski, M. X., Flygare, J. A., and Junutula, J. R. (2014) Site-specific trastuzumab maytansinoid antibody–drug conjugates with improved therapeutic activity through linker and antibody engineering. *J. Med. Chem.* 57, 7890–7899.
- (27) Zeglis, B. M., Davis, C. B., Aggeler, R., Kang, H. C., Chen, A., Agnew, B. J., and Lewis, J. S. (2013) An enzyme-mediated methodology for the site-specific radiolabeling of antibodies based on catalyst-free click chemistry. *Bioconjugate Chem.* 24, 1057–1067.
- (28) Raju, T. S., Briggs, J. B., Borge, S. M., and Jones, A. J. (2000) Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. *Glycobiology* 10, 477–486.
- (29) Ramakrishnan, B., Boeggeman, E., and Qasba, P. K. (2007) Novel method for in vitro O-glycosylation of proteins: Application for bioconjugation. *Bioconjugate Chem.* 18, 1912–1918.
- (30) Ramakrishnan, B., and Qasba, P. K. (2002) Structure-based design of beta 1,4-galactosyltransferase I (beta 4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: point mutation broadens beta 4Gal-T1 donor specificity. *J. Biol. Chem.* 277, 20833–20839.
- (31) Clark, P. M., Dweck, J. F., Mason, D. E., Hart, C. R., Buck, S. B., Peters, E. C., Agnew, B. J., and Hsieh-Wilson, L. C. (2008) Direct in-gel fluorescence detection and cellular imaging of O-GlcNAc-modified proteins. *J. Am. Chem. Soc.* 130, 11576–11582.
- (32) Khidekel, N., Arndt, S., Lamarre-Vincent, N., Lippert, A., Poulin-Kerstien, K. G., Ramakrishnan, B., Qasba, P. K., and Hsieh-Wilson, L. C. (2003) A chemoenzymatic approach toward the rapid and sensitive detection of O-GlcNAc posttranslational modifications. *J. Am. Chem. Soc.* 125, 16162–16163.
- (33) Sletten, E. M., and Bertozzi, C. R. (2009) Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew. Chem., Int. Ed.* 48, 6973–6998.
- (34) Agard, N. J., and Bertozzi, C. R. (2009) Chemical approaches to perturb, profile, and perceive glycans. *Acc. Chem. Res.* 42, 788–797.
- (35) Ning, X., Guo, J., Wolfert, M. A., and Boons, G. J. (2008) Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast huisgen cycloadditions. *Angew. Chem., Int. Ed.* 47, 2253–2255.
- (36) Wolfe, C. A., and Hage, D. S. (1995) Studies on the rate and control of antibody oxidation by periodate. *Anal. Biochem.* 231, 123–130.
- (37) Clamp, J. R., and Hough, L. (1966) Some observations on the periodate oxidation of amino compounds. *Biochem. J.* 101, 120–126.

(38) Carrasquillo, J. A., Pandi-Taskar, N., O'Donoghue, J. A., Humm, J. L., Zanzonico, P., Smith-Jones, P. M., Divgi, C. R., Pryma, D. A., Ruan, S., Kemeny, N. E., Fong, Y., Wong, D., Jaggi, J. S., Scheinberg, D. A., Gonen, M., Panageas, K. S., Ritter, G., Jungbluth, A. A., Old, L. J., and Larson, S. M. (2011) ^{124}I -huA33 antibody PET of colorectal cancer. *J. Nucl. Med.* 52, 1173–1180.

(39) Deri, M. A., Zeglis, B. M., Francesconi, L. C., and Lewis, J. S. (2013) PET imaging with ^{89}Zr : From radiochemistry to the clinic. *Nucl. Med. Biol.* 40, 3–14.

(40) Zeglis, B. M., Houghton, J. L., Evans, M. J., Viola-Villegas, N. T., and Lewis, J. S. (2014) Underscoring the influence of inorganic chemistry on nuclear imaging with radiometals. *Inorg. Chem.* 53, 1890–1899.

Supporting Information

“A Chemoenzymatic Strategy for the Synthesis of Site-Specifically Labeled Immunoconjugates for Multimodal PET and Optical Imaging”

Brian M. Zeglis¹, Charles B. Davis¹, Dalya Abdel-Atti¹, Sean D. Carlin¹, Aimei Chen², Robert Aggeler², Brian J. Agnew², and Jason S. Lewis^{1,*}

Table of Contents

Reagents and General Procedures.....	2
Cell Culture.....	2
Xenograft Models.....	2
Synthesis of <i>N</i> -Azidoacetylglactosamine.....	2
Synthesis of Alexa Fluor [®] 680-DIBO.....	2
Site-Specific Modification of huA33.....	2-3
Determination of Degree of Fluorescent Labeling.....	3
SDS-PAGE Analysis of the Antibody Conjugation.....	3
PNGaseF Treatment of Antibody.....	3
Non-specific Antibody Modification of DFO- ^{ns} huA33-Dye680.....	3
Radiolabeling of Antibody Constructs with ⁸⁹ Zr.....	4
Determination of Chelate Number.....	4
Radiolabeled Antibody Stability Assays.....	4
Immunoreactivity Assays.....	4
PET Imaging.....	4
Acute Biodistribution.....	5
Fluorescence Imaging.....	5
<i>Ex Vivo</i> Autoradiography and Fluorescence Microscopy.....	5
Statistical Analysis.....	5
Supplementary Figure S1.....	6
Supplementary Figure S2.....	6
Supplementary Figure S3.....	7
Supplementary Figure S4.....	7
Supplementary Table S1.....	8
Supplementary Table S2.....	8
References.....	9

Reagents and General Procedures: Unless otherwise noted, all chemicals were acquired from Sigma-Aldrich (St. Louis, MO) and were used without further purification. All water employed was ultra-pure ($>18.2 \text{ M}\Omega\text{cm}^{-1}$ at 25°C), all DMSO was of molecular biology grade ($>99.9\%$), and all other solvents were of the highest grade commercially available. Humanized A33 (huA33) was obtained courtesy of the Ludwig Institute for Cancer Immunotherapy. The GalT(Y289L) enzyme, UDP-GalNAz, and DIBO-DFO with a PEG linker were purchased from Thermo Fisher Scientific (Eugene, OR). All instruments were calibrated and maintained in accordance with standard, manufacturer-recommended quality-control procedures. UV-Vis measurements were taken using a Thermo Scientific NanoDrop 2000 Spectrophotometer.

^{89}Zr was produced at Memorial Sloan Kettering Cancer Center using 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. ^{89}Zr was purified in accordance with previously reported methods to create ^{89}Zr with a specific activity of $5.3 - 13.4 \text{ mCi}/\mu\text{g}$ ($195 - 497 \text{ MBq}/\mu\text{g}$).¹ A Capintec CRC-15R Dose Calibrator (Capintec, Ramsey, NJ) was used for all activity measurements. To ensure the accurate quantification of activity levels, a calibrated Perkin Elmer (Waltham, MA) Automatic Wizard² Gamma Counter was employed, and all experimental samples were counted for at least 1 min. Silica-gel impregnated glass-fiber instant thin-layer chromatography paper (Pall Corp., East Hills, NY) were used for radioTLC experiments, and all radioTLC strips were analyzed on a Bioscan AR-2000 radio-TLC plate reader using Winscan Radio-TLC software (Bioscan Inc., Washington, D. C.). All experiments performed on laboratory animals were performed according to a protocol approved by the Memorial Sloan-Kettering Institutional Animal Care and Use Committee (protocol 08-07-013).

Cell Culture: Human colorectal cancer cell line SW1222 was obtained from Sigma-Aldrich and maintained in Iscove's Modified Dulbecco's Medium, supplemented with 10% heat-inactivated fetal calf serum, 2.0 mM glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin in a 37°C environment containing 5% CO_2 . Cell lines were harvested and passaged weekly using a formulation 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 nude female (Hsd: Athymic Nude-nu) mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in ventilated cages, were given food and water *ad libitum*, and were allowed to acclimatize for approximately 1 week prior to inoculation. SW1222 tumors were induced on the right shoulder by a subcutaneous injection of 5.0×10^6 cells in a $150 \mu\text{L}$ cell suspension of a 1:1 mixture of fresh media: BD Matrigel (BD Biosciences, Bedford, MA). The xenografts reached ideal size for imaging and biodistribution ($\sim 100\text{-}150 \text{ mm}^3$) in approximately 15-18 d.

Synthesis of N-Azidoacetylgalactosamine (UDP-GalNAz): UDP-GalNAz was synthesized according to previously reported methods.^{2,3}

Synthesis of Alexa Fluor[®] 680-DIBO (DIBO-Dye680): To a solution of Alexa Fluor[®] 680 (Dye680) carboxylic acid, succinimidyl ester bis(triethylammonium salt) (14 mg, $11 \mu\text{mol}$) and N-(2-aminoethyl)-2-(5,6-dihydro-11,12-didehydro-dibenzo[a,e]cycloocten-5-yloxy)acetamide (DIBO amine, 4 mg, $12 \mu\text{mol}$) (Thermo Fisher Scientific, Eugene, OR) in 1 mL of anhydrous DMF was added triethylamine ($25 \mu\text{L}$, $180 \mu\text{mol}$) and the mixture was stirred at room temperature for 1 hour. The resulting reaction mixture was added into 10 mL of ethyl acetate slowly over 1 minute period while stirring vigorously at room temperature. The resulting precipitate was collected by centrifugation to give the desired product (DIBO-Dye680, 14 mg, 88 % yield) as a blue solid. TLC (silica gel, 15 % H_2O in CH_3CN): $R_f = 0.50$

Site-specific modification of huA33:

Glycans Modification: huA33 (2.5 mg, 5 mg/mL) underwent a buffer exchange into pre-treatment buffer (50 mM Bis-Tris, 100 mM NaCl, pH 6.0) using a micro-spin column (Amicon Ultra Centrifugal Filter 0.5 mL w/ $50,000 \text{ MWCO}$). The column was first equilibrated in 50 mM Bis-Tris, 100 mM NaCl, pH 6.0, and then spun for 5 minutes at $5000 \times g$. $500 \mu\text{L}$ of the huA33 antibody solution was added and then spun down for 5 minutes at $5000 \times g$. This procedure was repeated 3 times to complete the buffer exchange to 50 mM Bis-Tris, 100 mM NaCl, pH 6.0. After the final spin, the antibody was isolated in $\sim 110\text{-}120 \mu\text{L}$ of buffer. This solution was supplemented with $15 \mu\text{L}$ of β -

1.4-galactosidase [from *S. pneumonia* (2 mU/μL), obtained from Prozyme, Inc., Hayward, CA] and placed in an incubator at 37 °C for 6 h.

GalNAz Labeling: After the β-1.4-galactosidase treatment, 75 μL H₂O, 12.5 μL 1 M Tris buffer pH 7.6, 25 μL GalT(Y289L) (from a stock of 2 mg/mL in 50 mM Tris, 5 mM EDTA, pH 8), 2.5 μL 1 M MnCl₂ (in 0.1 M HCl), and 10 μL UDP-GalNAz (from a stock of 40 mM in H₂O) were added to the reaction solution to bring the final volume up to 250 μL. This reaction solution contained concentrations of 10 mg/mL huA33, 10 mM MnCl₂, 1.6 mM UDP-GalNAz, and 0.2 mg/mL GalT (Y289L) and was incubated for 16 h at 30 °C.

DIBO-DFO and DIBO-Dye680 Ligation: The solution from the GalNAz labeling step was purified via centrifugal filtration using 2 mL Amicon® Ultra centrifugal filters with a 50,000 Dalton molecular weight cut-off (Millipore Corp., Billerica, MA) and TBS buffer pH 7.4. After centrifugation, the modified huA33 antibody (1 mg in 900 μL TBS buffer pH 7.4) was combined with either 100 μL DIBO-DFO (from a 2 mM stock in DMSO), 100 μL DIBO-Dye680 (from a 2 mM stock in DMSO), or 100 μL of a mixture of the two DIBO constructs to yield a final solution containing 1 mg/mL huA33 and 0.2 mM DIBO. This solution was then incubated for 16 h at 25 °C.

Purification: After the click labeling, the completed antibody was purified via size exclusion chromatography (PD10 column, GE Healthcare) and concentrated via centrifugal filtration using 2 mL Amicon® Ultra centrifugal filters with a 50,000 Dalton molecular weight cut-off (Millipore Corp., Billerica, MA).

Determination of Degree of Fluorescent Labeling: To determine the degree of labeling (DOL) of the fluorophore-labeled antibodies, UV-Vis absorbance measurements were taken at 280 nm and 774 nm for three separate antibody concentrations. The degree of labeling was calculated using the following formulas:

$$A_{\text{huA33}} = A_{280} - A_{\text{max}}(\text{CF})$$

$$\text{DOL} = [A_{\text{max}} * \text{MW}_{\text{huA33}}] / [[\text{mAb}] * \epsilon_{\text{Alexa Fluor}^{\text{®}} 680}]$$

where the correction factor (CF) for Alexa Fluor[®] 680 was given as 0.05 by the supplier, MW_{huA33} = 150,000, ε₆₈₀, Alexa Fluor[®] 680 = 180,000, and ε_{280, huA33} = 210,000.

SDS-PAGE Analysis of the Antibody Conjugation: 1 μg antibody (2 μL of a 0.5 mg/mL stock) was combined with 18.5 μL H₂O, 3 μL 500 mM dithiothreitol (NuPAGE® 10X Sample Reducing Agent, Life Technologies), and 7.5 μL 4X electrophoresis buffer (NuPAGE® LDS Sample Buffer, Thermo Fisher, Eugene, OR). This mixture was then denatured by heating to 90 °C for 15 min using a heat block. Subsequently, 25 μL of each sample was then loaded alongside an appropriate molecular weight maker (Mark12™ unstained standard, Life Technologies) onto a 1 mm, 10 well 4-12% Bis-Tris protein gel (Life Technologies) and run for ~3 h at 10 V/cm in MOPS buffer. The completed gel was washed 3 times with H₂O, stained using SimplyBlue™ SafeStain (Life Technologies) for 1 h, and destained overnight in H₂O. The gel was then analyzed using an Odyssey Infrared Gel Scanner (Li-Cor Biosciences, Lincoln, NE).

PNGase F Treatment of Antibody: huA33 antibody construct (1 μg) in 10 μL TBS was denatured with 0.5% SDS and 40 mM DTT by adding 17 μL H₂O and 3 μL 10× Glycoprotein Denaturation Buffer (New England Biolabs, Ipswich, MA) and incubated for 10 min at 90 °C on a heat block. Subsequently, 18 μL H₂O, 6 μL 500 mM sodium phosphate, pH 7.5 (G7 reaction buffer from New England Biolabs), and 6 μL 10% NP-40 were added. This solution was then split into two aliquots: one aliquot was supplemented with 1 μL PNGase F (New England Biolabs) and incubated overnight at 37 °C, and the other aliquot was not treated. After incubation, SDS-PAGE electrophoresis was employed to analyze the PNGaseF-treated and untreated samples.

Non-specific Antibody Modification of DFO-^hhuA33-Dye680: huA33 (2 mg; 13 nmol) was dissolved in 1 mL of phosphate buffered saline (pH 7.4), and the pH of the solution was adjusted to 8.8-9.0 with NaHCO₃ (0.1 M). In parallel, a solution of DFO-NCS in DMSO (5-10 mg/mL) and Alexa Fluor 680-NHS in DMSO (5-10 mg/mL) were prepared. Volumes of these two solutions corresponding to 5 molar equivalents of DFO-NCS (65 nmol) and 4 molar equivalents of Alexa Fluor 680-NHS (52 nmol) were mixed together in a microcentrifuge tube. The pH-adjusted antibody solution was then added to the tube containing DFO-NCS and Alexa Fluor[®] 680-NHS, and the contents were mixed via vigorous pipetting. The resulting solution was incubated with gentle shaking for 60 min at 37 °C.

After 60 min, the modified antibody was purified using centrifugal filter units with a 50,000 molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp., Billerica, MA) and phosphate buffered saline (PBS, pH 7.4).⁴

Radiolabeling of Antibody Constructs with ⁸⁹Zr: For each antibody construct, 1 mg of immunoconjugate was added to 400 µL buffer (PBS, pH 7.4). [⁸⁹Zr]Zr-oxalate (3000-3250 µCi) in 1.0 M oxalic acid was adjusted to pH 7.0-7.5 with 1.0 M Na₂CO₃. After the evolution of CO₂(g), the ⁸⁹Zr solution was added to the antibody solution, and the resulting solution was incubated at room temperature for 1 h. After 1 h, the reaction progress was assayed using radio-TLC and an eluent of 50 mM EDTA (pH 5). Subsequently, the reaction was quenched with 50 µL of 50 mM EDTA (pH 5), and the antibody construct was purified using size-exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 500 µL fractions of PBS, pH 7.4) and concentrated via centrifugal filtration units with a 50,000 molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp., Billerica, MA). The radiochemical purity of the crude and final radiolabeled bioconjugate was assayed by radio-TLC again using 50 mM EDTA (pH 5.0) as an eluent. In the ITLC experiments, free ⁸⁹Zr⁴⁺ cations and [⁸⁹Zr]-EDTA elute with the solvent front, while radiolabeled antibody construct remains at the baseline.

Determination of Chelate Number: Radiometric isotopic dilution assays similar to those described in Anderson, *et al.* and Holland, *et al.*^{5, 6} were performed in order to determine the number of accessible DFO chelators appended per antibody. All experiments were performed in triplicate.

Radiolabeled Antibody Stability Assays: The stability of the site-specifically and non-site-specifically labeled radioimmunoconjugates with respect to radiochemical purity and loss of radioactivity from the antibody was investigated via incubation of the antibodies in human serum for 7 days at 37 °C (n = 3). After the prescribed incubation time, the radiochemical purity of the antibodies was determined via radio-TLC with an eluent of 50 mM EDTA pH 5.0.

Immunoreactivity Assays: The immunoreactivity of the ⁸⁹Zr-labeled radioimmunconjugates was determined using an antigen saturation assay. Briefly, suspensions of 1 × 10⁷ SW1222 colorectal cancer cells in 100 µL media were prepared in microcentrifuge tubes. In parallel, a solution of 0.1-0.4 ng/µL radioimmunoconjugate was prepared in PBS supplemented with 1% bovine serum albumin. 20 µL of the radioimmunoconjugate solution was then added to the cell suspension, and the resulting mixture was agitated via vigorous pipetting and subsequently incubated on ice for 1 h. After 1 h, the cells were then pelleted via centrifugation (600 g for 5 min). After centrifugation, the media was carefully removed and placed in a separate tube, and 1 mL of ice-cold PBS was added to the original tube containing the cells. The cells were then pelleted again via centrifugation (600 g for 5 min). Following centrifugation, the PBS was removed and placed in a separate tube. These PBS washing steps were repeated two more times. After washing, the amount of radioactivity in all five tubes — cell pellet, media, wash 1, wash 2, and wash 3 — was assayed using a gamma counter. The counting data was then background corrected, and the immunoreactive fraction was then calculated by dividing the counts in the cell pellet by the sum of the counts in the cell pellet, media, and three washes. No weighting was applied to the data, and the data were obtained in triplicate. Negative controls for non-specific radioimmunoconjugate binding to the microcentrifuge tube and binding of the radioimmunoconjugates to non-antigen-expressing cells were performed.

PET Imaging: PET imaging experiments were conducted on a microPET Focus rodent scanner (Concorde Microsystems). Mice bearing subcutaneous SW1222 xenografts (right shoulder; 100-150 mm³) were administered ⁸⁹Zr-DFO-^{ss}huA33-Dye680 or ⁸⁹Zr-DFO-^{ns}huA33-Dye680 (180-200 µCi, 72-80 µg, in 200 µL 0.9% sterile saline) via tail vein injection (t = 0). Approximately 5 minutes prior to the 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/gas mixture. PET data for each mouse were recorded via static scans at time points between 24 and 120 h. A minimum of 60 million coincident events were recorded for each scan, which lasted between 5-20 min. An energy window of 350-700 keV and a coincidence timing window of 6 ns were used. Data were sorted into 2-dimensional histograms by Fourier re-binning, and transverse images were reconstructed by filtered back-projection (FBP) into a 128 × 128 × 63 (0.72 × 0.72 × 1.3 mm) matrix. Image data were normalized to correct for dead-time count losses, positron-branching ratio, physical decay to the time of injection, and non-uniformity of response of the PET. No scatter, partial-volume averaging, or scatter correction was applied. A system calibration factor derived from the imaging of a mouse-sized water-equivalent phantom

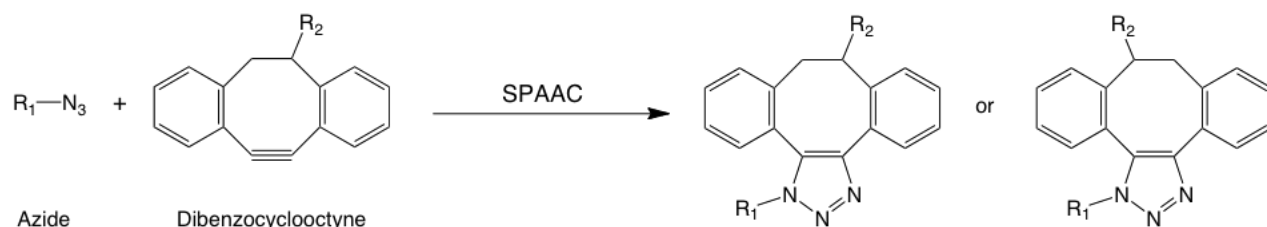
containing ^{89}Zr was used to convert the counting rates in the reconstructed images to activity concentrations (percentage injected dose [%ID] per gram of tissue). Images were analyzed using ASIPro VMTM software (Concorde Microsystems).

Acute Biodistribution: Athymic nude mice bearing subcutaneous SW1222 xenografts (right shoulder; 100-150 mm³) were randomized before the study and were warmed gently with a heat lamp for 5 min before the administration of ^{89}Zr -DFO-^{ss}huA33-Dye680 (15-20 μCi , 6-8 μg , in 200 μL 0.9% sterile saline) via tail vein injection ($t = 0$). Subsequently, the mice ($n = 4$ per group) were euthanized by $\text{CO}_2(\text{g})$ asphyxiation at 48 and 96 h post-injection, and 13 tissues (including tumor) were removed, washed, dried, weighed, and counted in a gamma counter. The number of counts in each tissue was background and decayed corrected to the time of injection and converted to activity units (μCi) using a calibration curve generated from known standards. The %ID/g for each tissue sample was then calculated by normalization to the total activity injected and the mass of each tissue. In addition, in order to assay antigen specificity, an additional cohort of animals ($n = 4$) were administered ^{89}Zr -DFO-^{ss}huA33-Dye680 with significantly lowered specific activity (LSA) — achieved by co-injection of the standard dose of ^{89}Zr -DFO-^{ss}huA33-Dye680 along with 300 μg of the cold, unlabeled DFO-^{ss}huA33-Dye680 conjugate — and were euthanized 72 h post-injection, and analyzed in a manner identical to the other cohorts in the study.

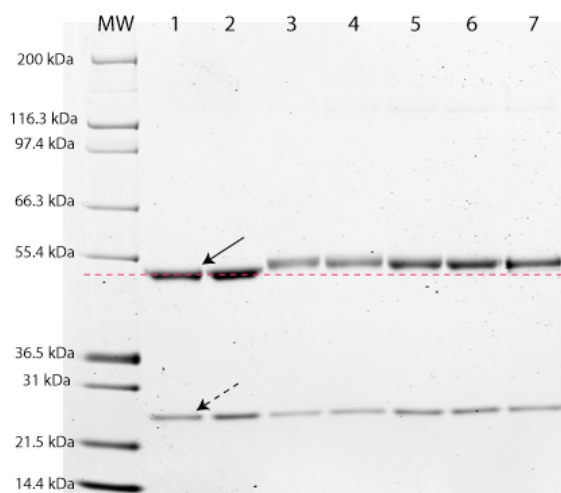
Fluorescence Imaging: Mice bearing subcutaneous SW1222 xenografts (right shoulder; 100-150 mm³) were administered ^{89}Zr -DFO-^{ss}huA33-Dye680 or ^{89}Zr -DFO-^{ns}huA33-Dye680 (72-80 μg , in 200 μL 0.9% sterile saline) via tail vein injection ($t = 0$). Approximately 5 minutes prior to the fluorescence 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/gas mixture. Near-infrared fluorescent images were acquired with an IVIS Spectrum Preclinical Imaging System (Perkin Elmer) using excitation/emission settings of 640nm/704nm. Images were processed using Living Image (v4.4) and values are reported as radiant efficiency to allow direct comparison of images from each experiment. All region-of-interest measurements were generated via automated analysis to avoid user bias.

Ex Vivo Autoradiography and Fluorescence Microscopy Following final imaging, tumors were excised, embedded in OCT, and snap-frozen. Series of sequential 1 mm thick cryosections were cut and immediately exposed to a phosphor plate for determination of ^{89}Zr distribution. Digital autoradiographic images at 50 μm pixel size were obtained as previously described.⁷ Following autoradiography, sections were fixed in 4% paraformaldehyde. Alexa Fluor 680 images were acquired using an Zeiss Axioplan2 fluorescence microscope connected to a CCD camera, equipped with a motorized stage (Prior Scientific Instruments, Cambridge, UK, USA) and MetaMorph software (Molecular Devices, Sunnyvale, CA) allowing the individual captured image frames to be rendered into a montage of the entire tumor section. Finally, hematoxylin and eosin staining was performed to visualize tumor morphology, and images were acquired in a similar manner. Montage images of each stain, all acquired from the same tumor section, were manually registered using Photoshop CS5 software (Adobe Systems). Image rebinning and pixel-by-pixel correlations were carried out as previously described.⁸

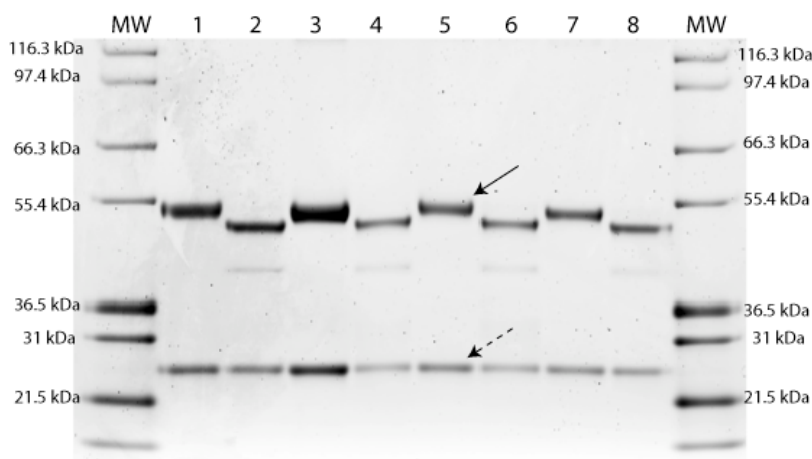
Statistical Analysis: Data were analyzed by the unpaired, two-tailed Student's t-test. Differences at the 95% confidence level ($P < 0.05$) were considered to be statistically significant.



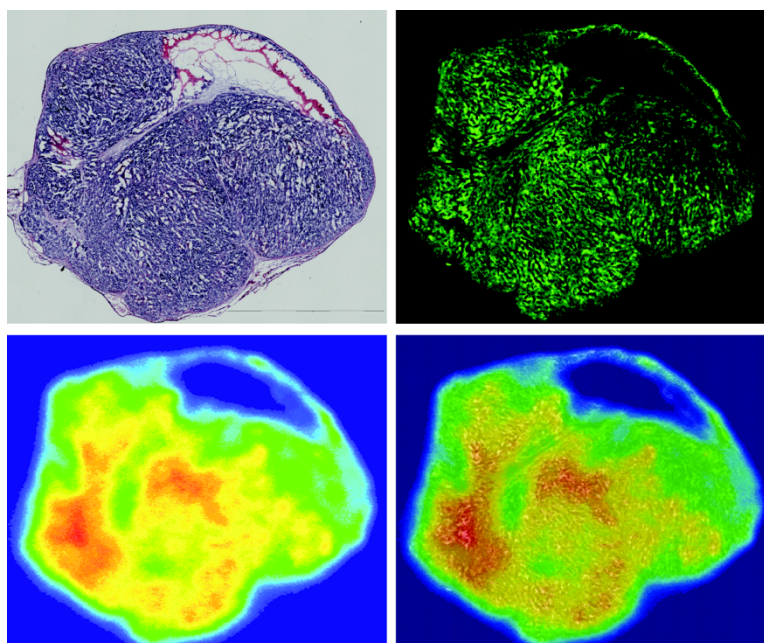
Supplementary Figure S1. Diagram of the strain-promoted azide-alkyne cycloaddition



Supplementary Figure S2. SDS-PAGE analysis of site-specifically labeled immunoconjugates. Lane 1: huA33. Lane 2: ^{ss}huA33-N₃. Lane 3: ^{ss}huA33-DFO. Lanes 4, 5, and 6: DFO-^{ss}huA33-Dye680 synthesized with 3:1, 1:1, and 1:3 mixtures of DIBO-DFO:DIBO-Dye680, respectively. Lane 7: ^{ss}huA33-Dye680. The solid and dotted arrows indicate the antibody heavy chain and light chain, respectively, and the red dotted line indicates the molecular weight of the huA33 heavy chain. MW = molecular weight ladder.



Supplementary Figure S3. SDS-PAGE illustrating the treatment of site-specifically labeled immunoconjugates with PNGase F. Lane 1: untreated huA33. Lane 2: huA33 treated with PNGaseF. Lane 3: untreated ^{88}Sg huA33- N_3 . Lane 4: ^{88}Sg huA33- N_3 treated with PNGaseF. Lane 5: untreated ^{88}Sg huA33-DFO. Lane 6: ^{88}Sg huA33-DFO treated with PNGaseF. Lane 7: untreated ^{88}Sg huA33-Dye680. Lane 8: ^{88}Sg huA33-Dye680 treated with PNGaseF. The solid and dotted arrows indicate the antibody heavy chain and light chain, respectively, and the bands at ~40 kDa are the result of excess PNGaseF enzyme. MW = molecular weight ladder.



Supplementary Figure S4. Autoradiography, histology, and fluorescence microscopy of resected SW1222 xenografts from multimodality imaging experiment. Top left: hematoxylin and eosin staining. Top right: fluorescence microscopy indicating Alexa Fluor[®] 680 localization (green). Bottom left: autoradiography indicating ^{89}Zr localization. Bottom right: overlay of fluorescence microscopy and autoradiography.

	⁸⁹ Zr-DFO- ^{ss} huA33-Dye680		
	48 h	120 h	48 h LSA
Blood	5.7 ± 1.2	2.4 ± 1.7	9.7 ± 1.4
Tumor	45.9 ± 7.7	45.7 ± 8.1	14.7 ± 1.3
Heart	3.7 ± 1.8	3.2 ± 1.7	4.3 ± 0.6
Lung	3.7 ± 1.6	4.1 ± 2.4	3.7 ± 0.3
Liver	3.9 ± 2.3	2.4 ± 2.1	6.3 ± 1.1
Spleen	2.0 ± 1.1	5.1 ± 1.2	3.1 ± 0.4
Stomach	0.7 ± 0.3	0.9 ± 0.6	0.4 ± 0.4
Large Intestine	0.8 ± 0.2	0.5 ± 0.2	0.7 ± 0.1
Small Intestine	0.9 ± 0.3	1.1 ± 0.4	0.8 ± 0.2
Kidney	3.2 ± 1.8	3.8 ± 1.3	3.5 ± 0.9
Muscle	1.5 ± 0.3	1.4 ± 0.6	1.0 ± 0.3
Bone	2.8 ± 1.2	5.6 ± 1.4	6.9 ± 3.3

Supplementary Table S1. Acute biodistribution uptake values versus time for ⁸⁹Zr-DFO-^{ss}huA33-Dye680 in mice bearing subcutaneous SW1222 xenografts (n = 4 for each time point). Mice were administered the radioimmunoconjugate (0.55 – 0.75 MBq [15-20 µCi] in 200 µL 0.9% sterile saline) via tail vein injection (t = 0). For the 48 h time point, an additional cohort of animals was given radioimmunoconjugate with dramatically lowered specific activity (LSA), achieved by the co-injection of the standard dose of the ⁸⁹Zr-labeled construct mixed with 300 µg of cold, unlabeled DFO-^{ss}huA33-Dye680.

	⁸⁹ Zr-DFO- ^{ss} huA33-Dye680		
	48 h	96 h	48 h LSA
<i>Tumor:Blood</i>	8.1 ± 2.2	18.7 ± 13.5	1.5 ± 0.2
<i>Tumor:Heart</i>	12.3 ± 6.3	14.2 ± 7.9	3.4 ± 0.6
<i>Tumor:Lung</i>	12.5 ± 5.9	11.3 ± 6.9	3.9 ± 0.5
<i>Tumor:Liver</i>	11.6 ± 7.1	18.9 ± 16.4	2.3 ± 0.5
<i>Tumor:Spleen</i>	23.3 ± 13.6	8.9 ± 2.7	4.7 ± 0.7
<i>Tumor:Stomach</i>	67.4 ± 27.9	52.5 ± 37.4	35.4 ± 31.8
<i>Tumor:LI</i>	56.1 ± 18.9	88.5 ± 37.9	22.3 ± 3.1
<i>Tumor:SI</i>	53.4 ± 22.5	41.5 ± 17	17.7 ± 5.4
<i>Tumor:Kidney</i>	14.2 ± 8.4	11.9 ± 4.5	4.2 ± 1.2
<i>Tumor:Muscle</i>	30.1 ± 8.2	33.5 ± 15.6	14.7 ± 4.8
<i>Tumor:Bone</i>	16.5 ± 7.8	8.1 ± 2.5	2.1 ± 1

Supplementary Table S2. Tumor-to-tissue activity ratios versus time for ⁸⁹Zr-DFO-^{ss}huA33-Dye680 in mice bearing subcutaneous SW1222 xenografts (n = 4 for each time point). Mice were administered the radioimmunoconjugates (0.55 – 0.75 MBq [15-20 µCi] in 200 µL 0.9% sterile saline) via tail vein injection (t = 0). For the 48 h time point, an additional cohort of animals was given radioimmunoconjugate with dramatically lowered specific activity (LSA), achieved by the co-injection of the standard dose of the ⁸⁹Zr-labeled construct mixed with 300 µg of cold, unlabeled DFO-^{ss}huA33-Dye680.

References

- (1) Holland, J. P., Sheh, Y. C., and Lewis, J. S. (2009) Standardized methods for the production of high specific-activity zirconium-89. *Nucl. Med. Biol.* 36, 729-739.
- (2) Hang, H. C., Yu, C., Kato, D. L., and Bertozzi, C. R. (2003) A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation. *Proc. Nat. Acad. Sci. U.S.A.* 100, 14846-14851.
- (3) Laughlin, S. T., and Bertozzi, C. R. (2007) Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. *Nature Protoc.* 2, 2930-2944.
- (4) Vosjan, M., Perk, L. R., Visser, G. W. M., Budde, M., Jurek, P., Kiefer, G. E., and van Dongen, G. (2010) Conjugation and radiolabeling of monoclonal antibodies with zirconium-89 for PET imaging using the bifunctional chelate p-isothiocyanatobenzyl-desferrioxamine. *Nature Protoc.* 5, 739-743.
- (5) Anderson, C. J., Connett, J. M., Schwarz, S. W., Rocque, P. A., Guo, L. W., Philpott, G. W., Zinn, K. R., Meares, C. F., and Welch, M. J. (1992) Copper-64-labeled antibodies for PET imaging. *J. Nucl. Med.* 33, 1685-1691.
- (6) Holland, J. P., Caldas-Lopes, E., Divilov, V., Longo, V. A., Taldone, T., Zatorska, D., Chiosis, G., and Lewis, J. S. (2010) Measuring the pharmacodynamic effects of a novel Hsp90 inhibitor on HER2/neu expression in mice using Zr-89-DFO-trastuzumab. *Plos One* 5, e8859.
- (7) Carlin, S., Khan, N., Ku, T., Longo, V. A., Larson, S. M., and Smith-Jones, P. M. (2010) Molecular targeting of carbonic anhydrase IX in mice with hypoxic HT29 colorectal tumor xenografts. *PLoS ONE* 5, e10857.
- (8) Carlin, S., Pugachev, A., Sun, X., Burke, S., Claus, F., O'Donoghue, J., Ling, C. C., and Humm, J. L. (2009) In vivo characterization of a reporter gene system for imaging hypoxia-induced gene expression. *Nucl. Med. Biol.* 36, 821-831.