Journal of Visualized Experiments

Synthesis and Bioconjugation of Thiol-Reactive Reagents for the Creation of Site-Selectively Modified Immunoconjugates

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59063R1
Full Title:	Synthesis and Bioconjugation of Thiol-Reactive Reagents for the Creation of Site-Selectively Modified Immunoconjugates
Keywords:	Site-specific bioconjugation; site-selective bioconjugation; maleimide; thiol; sulfhydryl; radioimmunoconjugate; immunoconjugate
Corresponding Author:	Brian M. Zeglis, Ph.D. Memorial Sloan Kettering Cancer Center New York, NY UNITED STATES
Corresponding Author's Institution:	Memorial Sloan Kettering Cancer Center
Corresponding Author E-Mail:	bz102@hunter.cuny.edu
Order of Authors:	Maria Davydova
	Guillaume Dewaele Le Roi
	Pierre Adumeau
	Brian M. Zeglis, Ph.D.
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	New York, NY, USA

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Click here to access/download;Manuscript;59063_R1.docx

TITLE:
Synthesis and Bioconjugation of Thiol-Reactive Reagents for the Creation of Site-Selectively Modified Immunoconjugates

AUTHORS AND AFFILIATIONS:
Maria Davydova[1], Guillaume Dewaele Le Roi[1,2], Pierre Adumeau[1], Brian M. Zeglis[1,2,3,4]

[1] Department of Chemistry, Hunter College of the City University of New York, New York, NY, USA
[2] Ph.D. Program in Chemistry, The Graduate Center of the City University of New York, New York, NY, USA
[3] Department of Radiology, Memorial Sloan Kettering Cancer Center, New York, NY, USA
[4] Department of Radiology, Weill Cornell Medical College, New York, NY, USA

Corresponding Author:
Brian M. Zeglis
bz102@hunter.cuny.edu

Email Addresses of Co-Authors:
Maria Davydova (mariadavydova96@gmail.com)
Guillaume Dewaele Le Roi (gdewaeleleroi@gradcenter.cuny.edu)
Pierre Adumeau (pierre.adumeau@hotmail.fr)

KEYWORDS:
Site-specific bioconjugation; site-selective bioconjugation; maleimide; thiol; sulfhydryl; radioimmunoconjugate; immunoconjugate

SUMMARY:
In this protocol, we will describe the synthesis of PODS, a phenyoxadiazolyl methyl sulfone-based reagent for the site-selective attachment of cargos to the thiols of biomolecules, particularly antibodies. In addition, we will describe the synthesis and characterization of a PODS-bearing bifunctional chelator and its conjugation to a model antibody.

ABSTRACT:
Maleimide-bearing bifunctional probes have been employed for decades for the site-selective modification of thiols in biomolecules, especially antibodies. Yet maleimide-based conjugates display limited stability in vivo because the succinimidyl thioether linkage can undergo a retro-Michael reaction. This, of course, can lead to the release of the radioactive payload or its exchange with thiol-bearing biomolecules in circulation. Both of these processes can produce elevated activity concentrations in healthy organs as well as decreased activity concentrations in target tissues, resulting in reduced imaging contrast and lower therapeutic ratios. In 2018, we reported the creation of a modular, stable, and easily accessible phenyloxadiazolyl methyl sulfone reagent — dubbed 'PODS' — as a platform for thiol-based bioconjugations. We have clearly demonstrated that PODS-based site-selective bioconjugations reproducibly and robustly

create homogenous, well-defined, highly immunoreactive, and highly stable radioimmunoconjugates. Furthermore, preclinical experiments in murine models of colorectal cancer have shown that these site-selectively labeled radioimmunoconjugates exhibit far superior in vivo performance compared to radiolabeled antibodies synthesized via maleimide-based conjugations. In this protocol, we will describe the four-step synthesis of PODS, the creation of a bifunctional PODS-bearing variant of the ubiquitous chelator DOTA (PODS-DOTA), and the conjugation of PODS-DOTA to the HER2-targeting antibody trastuzumab.

INTRODUCTION:

Radiopharmaceutical chemists have long exploited the selectivity and specificity of antibodies for biomarkers of disease for both nuclear imaging and targeted radiotherapy[1]. Far and away the most common approach to the radiolabeling of antibodies is predicated on the indiscriminate attachment of radiolabeled prosthetic groups or radiometal chelators to amino acids — most often lysines — within the structure of the immunoglobulin (**Figure 1A**)[2]. While this strategy is certainly effective, its random, non-site-specific nature can create problems. Specifically, traditional bioconjugation approaches produce poorly-defined and heterogeneous immunoconjugates composed of mixtures of thousands of different regioisomers, each with its own set of biological and pharmacological properties[3]. Furthermore, random bioconjugation can impede the immunoreactivity of antibodies if the cargo is appended to the immunoglobulin's antigen-binding domains.

Over the years, a variety of site-specific and site-selective bioconjugation strategies have been developed in order to address these problems[4,5]. The most common of these approaches relies on the ligation of maleimide-bearing probes to the sulfhydryl groups of cysteines (**Figure 1B**). IgG1 antibodies naturally contain 4 inter-chain disulfide bridges, linkages that can be selectively reduced to yield free thiols capable of undergoing Michael addition reactions with maleimides to form succinimidyl thioether bonds. The use of thiols and maleimides is certainly an improvement over traditional methods, and a wide variety of maleimide-bearing synthons and bifunctional chelators are currently available. However, it is important to note that this methodology has serious limitations as well. Maleimide-based immunoconjugates exhibit limited stability in vivo because the thioether linkage can undergo a retro-Michael reaction (**Figure 2**)[6-10]. This, of course, can lead to the release of the radioactive payload or its exchange with thiol-bearing biomolecules in circulation (e.g., glutathione or serum albumin). Both of these processes can increase activity concentrations in healthy organs as well as decrease activity concentrations in target tissues, resulting in reduced imaging contrast and lower therapeutic ratios. Several alternative thiol-reactive reagents have been developed in an effort to circumvent these issues, including tosylates, bromo- and iodo-acetyls, and vinyl sulfones[11-17]. However, all of these approaches have limitations that have hampered their widespread application.

About five years ago, the laboratory of the late Carlos Barbas III at Scripps Research Institute pioneered the use of phenyloxadiazolyl methyl sulfones as reagents for the selective formation of highly stable linkages with thiols (**Figure 1C and Figure 3**)[18,19]. The authors employed a phenyloxadiazolyl methyl sulfone-bearing variant of fluorescein to modify several antibodies

engineered to contain free cysteine residues, ultimately producing immunoconjugates with higher stability than analogous constructs created using maleimide-based probes. Upon seeing this promising work, we were somewhat surprised that this technology had only been used scarcely in radiochemistry and had not yet been used at all in the synthesis of bifunctional chelators or radioimmunoconjugates[20,21]. This dearth of applications, however, soon began to make more sense: several attempts at procuring the reagent from Sigma-Aldrich resulted in the receipt of complex mixtures of degradation products with <15% of the desired compound. In addition, synthesizing the reported reagent ourselves was not a realistic option either, as the published synthetic route is somewhat cumbersome and requires sophisticated organic chemistry equipment that most radiochemistry and molecular imaging laboratories — including ours — simply do not possess.

In response to these obstacles, we set out to create an easily accessible and highly stable phenyloxadiazolyl methyl sulfone reagent that can be obtained via a robust and reasonably facile synthetic route. Earlier this year, we reported the creation of a modular, stable, and easily accessible phenyloxadiazolyl methyl sulfone reagent — dubbed 'PODS' — as a platform for thiol-based bioconjugations (**Figure 1C and Figure 3**)[22]. The key difference between PODS and the reagent reported by Barbas, et al. is that the former employs an aniline ring attached to the phenyloxadiazolyl methyl sulfone moiety, while the latter features a phenol in the same position (**Figure 4**). This change facilitates a more straightforward and accessible synthetic route as well as — if our experience with the commercially available compound is emblematic — a more stable final reagent. In this work, we also synthesized a pair of PODS-bearing bifunctional chelators — PODS-DFO and PODS-CHX-A″-DTPA — to facilitate the creation of ^{89}Zr- and ^{177}Lu-labeled radioimmunoconjugates, respectively. As we will discuss, we have demonstrated that PODS-based site-selective bioconjugations reproducibly and robustly create homogenous, well-defined, highly immunoreactive, and highly stable radioimmunoconjugates. Furthermore, preclinical experiments in murine models of colorectal cancer have shown that these site-selectively labeled radioimmunoconjugates exhibit superior in vivo performance compared to radiolabeled antibodies synthesized via maleimide-based conjugations.
The over-arching goal of this work is to facilitate the creation of well-defined, homogeneous, highly stable, and highly immunoreactive immunoconjugates for in vitro and in vivo applications. The synthetic approach is simple enough to be performed in almost any laboratory, and the parent PODS reagent can be modified with a plethora of different chelators, fluorophores, or cargoes. In this protocol and the accompanying video, we will describe the simple, four-step synthesis of PODS (**Figure 5**); the creation of a PODS-bearing variant of DOTA, a widely used chelator for the coordination of ^{64}Cu, ^{68}Ga, ^{111}In, ^{177}Lu, and ^{225}Ac (**Figure 6**); and the bioconjugation of PODS-DOTA to a model antibody, the HER2 targeting IgG1 trastuzumab (**Figure 7**).

PROTOCOL:

1. The synthesis of 4-[5-(methylthio)-1,3,4-oxadiazol-2-yl]-aniline (1)

NOTE: Due to the light-sensitivity of the compound, keep all reactions in foil-covered vessels.

1.1. In a 10 mL round bottom flask, dissolve 100 mg (0.517 mmol, 1 equivalent) of 5-(4-aminophenyl)-1,3,4-oxadiazole-2-thiol in 3 mL of methanol.

1.2. To this solution, add 360 µL of diisopropylethylamine (DIPEA; 2.07 mmol; 4 equivalents; anhydrous) and a small magnetic stir bar. Cover the flask with a rubber stopper and stir the solution for 10 minutes at room temperature.

1.3. Using a 1 mL glass syringe, poke a hole through the rubber stopper and quickly add 32 µL (0.517 mmol, 1 equivalent) of iodomethane to this mixture. Allow the mixture to react for 45 minutes at room temperature.

NOTE: Due to the potential harmful effects of iodomethane, this reaction should be done in a chemical fume hood.

1.4. Set the water bath of a rotary evaporator to 40 °C and slowly reduce the pressure to remove the solvent to afford a white solid.

1.5. Dissolve the solid in 3 mL of ethyl acetate and wash at least three times with a 5 mL solution of 0.1 M sodium carbonate using a separatory funnel.

NOTE: Periodically take spot-tests of the aqueous phase under a UV lamp; once nothing is seen under the lamp, you can stop the washes.

1.6. Collect the organic phase in a separatory funnel and wash it with water until the pH of the aqueous phase reaches 6.8-7.0 (using pH paper).

1.7. Collect the organic phase and add magnesium sulfate to remove any traces of water.

NOTE: The magnesium sulfate should be added with a small spatula, after which the solution should be swirled. If fine particles of the drying agent are still seen, the solution is dry. If not, add small amounts of magnesium sulfate until fine particles can be seen.

1.8. Filter the mixture using a medium glass frit or filter paper.

1.9. Evaporate the volatiles using a rotary evaporator, a process which should produce the desired product as white needles.

2. The synthesis of tert-butyl[18-({4-[5-(methylthio)-1,3,4-oxadiazol-2-yl]phenyl}amino)-15,18-dioxo-4,7,10-trioxa-14-azaoctadecyl] carbamate (2)

NOTE: Due to the light-sensitivity of the compound, keep all reactions in foil-covered vessels.

2.1. In a 25 mL round bottom flask, dissolve 387 mg (0.92 mmol, 1.0 equivalent) of NBoc-N'-

succinyl-4,7,10-trioxa-1,13-tridecanediamine in 10 mL of dichloromethane.

2.2. To this solution, add 480 µL (2.76 mmol, 3 equivalents) of DIPEA, 264 mg (1.38 mmol; 1.5 equivalents) of N-ethyl-N'-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI), and 200 mg (0.97 mmol, 1.1 equivalents) of **1**. Seal the vessel with a glass stopper and let the reaction stir for 5 days at room temperature.

NOTE: Be mindful of the evaporation of dichloromethane. If needed, add more throughout the week.

2.3. Wash the mixture in a separatory funnel with a solution of 1 M hydrochloric acid (3 x 5 mL).

2.4. Collect the organic phase and continue to wash it in a separatory funnel, first with a solution of 1 M Na_2CO_3 (2 x 5 mL) and then with water (3 x 5 mL).

2.5. Collect the organic phase and add magnesium sulfate to remove any traces of water (see Step 1.7). Filter the mixture using a medium glass frit or filter paper.

2.6. Using a rotary evaporator, remove the volatile solvents under reduced pressure to afford an off-white solid.

2.7. Re-dissolve this solid in 10 mL of ethyl acetate and precipitate the product via the gradual (e.g., 2 mL at a time) addition of 30 mL of cyclohexane.

2.8. Filter the solution with filter paper or a medium glass frit to obtain the product as a white powder.

3. The synthesis of tert-butyl[18-({4-[5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl]phenyl}amino)-15,18-dioxo-4,7,10-trioxa-14-azaoctadecyl] carbamate (3)

NOTE: Due to the light-sensitivity of the compound, keep all reactions in foil-covered vessels.

3.1. In a 10 mL round-bottom flask, dissolve 30 mg (0.05 mmol; 1 equivalent) of **2** in 4 mL of dichloromethane.

3.2. Slowly add in 49 mg (0.2 mmol; 4 equivalents) of 70% m-chloroperbenzoic acid to this mixture and cover the reaction vessel with a glass stopper. Stir the solution overnight at room temperature, ultimately yielding a yellow mixture.

3.3. Wash the yellow mixture in a separatory funnel, first with a 0.1 M solution of NaOH (3 x 5 mL) and then with water (3 x 5 mL).

3.4. Dry the organic phase with magnesium sulfate and filter the mixture using a medium glass frit or filter paper.

3.5. Using a rotary evaporator, remove the solvents under reduced pressure to obtain the product as a pale solid.

4. The synthesis of N^1-(3-{2-[2-(3-aminopropoxy)ethoxy]-ethoxy}propyl)-N^4-{4-[5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl] phenyl} succinamide (PODS)

4.1. In a 25 mL round bottom flask, dissolve 30 mg of **3** in 2.0 mL of dichloromethane.

4.2. Add 400 µL of trifluoroacetic acid and seal the flask with a glass stopper.

4.3. Stir the reaction mixture at room temperature for 3 hours.

4.4. Using a rotary evaporator, remove the volatiles under reduced pressure at room temperature, leaving an oily residue.

4.5. Dissolve the oily residue in 7 mL of water and, using a separatory funnel, wash with ethyl acetate (3 x 4 mL). Keep the aqueous layer.

4.6. Lyophilize the aqueous layer to afford **PODS** as a white powder.

NOTE: The molar absorption coefficients for PODS at 280 and 298 nm are 9,900 and 12,400 $cm^{-1}M^{-1}$, respectively.

5. The synthesis of PODS-DOTA

5.1. In a 1.5 mL microcentrifuge tube, dissolve 10 mg of PODS in 300 µL of dimethyl sulfoxide (0.018 mmol; 1 equivalent) and add 26 µL of N,N-diisopropylethylamine (0.15 mmol; 8 equivalents).

5.2. Dissolve 15.2 mg of DOTA-Bn-NCS (0.02 mmol; 1.2 equivalents) in 100 µL of dimethylsulfoxide and combine this solution with the solution from Step 5.1. Seal the microcentrifuge tube.

5.3. Allow the reaction to incubate overnight at room temperature.

5.4. Purify the product using reverse-phase C_{18} HPLC chromatography to remove any unreacted DOTA-Bn-NCS.

NOTE: Retention times are obviously highly dependent on the HPLC equipment of each laboratory (pumps, columns, tubing, etc.), and appropriate controls should be run prior to purification. However, to present an example, if a gradient of 5:95 MeCN/H_2O (both with 0.1% TFA) to 70:30 MeCN/H_2O (both with 0.1% TFA) over 30 min, a semi-preparative 19 x 250 mm

C_{18} column, and a flow rate of 6 mL/min are used, PODS, p-SCN-Bn-DOTA, and PODS-DOTA will have retention times of around 14.4, 18.8, and 19.6 min, respectively. All three compounds can be monitored at 254 nm.

6. The bioconjugation of PODS-DOTA to trastuzumab

NOTE: For this step, we started with a 16.4 mg/mL stock solution of trastuzumab.

6.1. In a low protein binding 1.5 mL microcentrifuge tube, dilute 61 µL of the trastuzumab stock solution (1 mg; 6.67 nmol, 1 equivalent) with 859 µL of phosphate buffered saline (pH 7.4).

6.2. To this mixture, add 6.7 µL of a freshly made 10 mM solution of TCEP in H_2O (66.7 nmol, 10 equivalents).

6.3. Prepare a 1 mg/mL solution of PODS-DOTA in DMSO and add 73 µL of this PODS-DOTA solution to the reaction mixture (66.67 nmol, 10 equivalents).

6.4. Seal the microcentrifuge tube and incubate the solution for 2 hours at room temperature.

6.5. After 2 hours, purify the immunoconjugate using a pre-packed disposable size exclusion desalting column.

6.5.1. First, equilibrate the size exclusion column as described by the supplier to remove any preservatives present in the column during storage. A typical procedure involves washing the column 5 times with a volume of PBS that corresponds to the volume of the column: 5 x 2.5 mL of PBS.

6.5.2. Next, add the reaction mixture to the size exclusion column noting the volume of the reaction mixture.

6.5.3. After the reaction mixture has entered the column, add an appropriate amount of PBS to bring the total volume of solution added to the column up to 2.5 mL. For example, if the conjugation reaction resulted in a total volume of 1.3 mL, 1.2 mL of additional PBS would need to be added to the column.

6.5.4. Finally, collect the product using 2 mL of PBS as the eluent.

6.6. Concentrate the final immunoconjugate with centrifugal filtration units with a 50 kDa molecular weight cut-off.

REPRESENTATIVE RESULTS:

The first four steps of this protocol — the synthesis of PODS — have been designed to be robust and reliable. The deprotonation and substitution of 5-(4-aminophenyl)-1,3,4-oxadiazole-2-thiol to form the desired thioether product affords the thioether in >99% yield after just 45

minutes. Next, the ligation between **1** and N-Boc-N'-succinyl-4,7,10-trioxa-1,13-tridecanediamine was achieved via a standard peptide coupling procedure, resulting in the collection of the product (**2**) in 55% yield. Then, the oxidation of **2** was performed using m-chloroperoxybenzoic acid, a widely used oxidant. Following the washing steps, **3** was obtained as a pale solid in ~90% yield. Finally, the removal of the tert-butyloxycarbonyl protecting group from **3** was done according to standard procedures, using a 4:1 ratio of dichloromethane:trifluoroacetic acid. After the lyophilization of the aqueous phase, our product — PODS — was obtained as a white powder in 98% yield. The progress of the reaction was followed via thin layer chromatography, and the identity of each product was confirmed via ^{1}H-NMR, ^{13}C-NMR, and HRMS-ESI (**Table 1**).

One of the principal advantages of the PODS reagent is its modularity. A variety of chelators, fluorophores, toxins, or other cargoes can be appended to the compound's pendant amine. In the protocol at hand, we are using the ubiquitous chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) as a representative payload. DOTA, of course, has been used in a wide range of biomolecular radiopharmaceuticals as a chelator for radiometals including ^{68}Ga, ^{64}Cu, ^{111}In, ^{90}Y, ^{177}Lu, and ^{225}Ac. To this end, an isothiocyanate-bearing variant of DOTA (p-SCN-Bn-DOTA) was employed and coupled to the pendant amine of PODS via straightforward coupling conditions. The resultant bifunctional chelator was then purified via reverse phase C_{18} HPLC and isolated in ~75% yield. As with the other precursors, the progress of the reaction was followed via thin layer chromatography, and the identity of the product was confirmed via ^{1}H-NMR, ^{13}C-NMR, and HRMS-ESI (**Table 1**).

In the final step of the protocol, we discuss the site-selective bioconjugation of PODS-DOTA to a model immunoglobulin, the HER2-targeting antibody trastuzumab. To this end, the disulfide linkages of the antibody's hinge region are selectively reduced with the reducing agent TCEP [tris(2-carboxyethyl)phosphine]. Following this reduction step, the antibody is incubated with PODS-DOTA for 2 h at room temperature and subsequently purified via size exclusion chromatography. In this case, the purified, DOTA-bearing immunoconjugate was obtained in ~80% yield, and MALDI-ToF analysis revealed a degree of labeling (DOL) of ~1.8 DOTA/mAb. Generally speaking, we have found that 10 equivalents of TCEP, 10 equivalents of the PODS reagent, and a 2 h incubation are sufficient to yield an immunoconjugate with a DOL of 2 PODS/mAb (**Table 2**). This result remains consistent across a range of human, humanized, and chimeric IgG1 antibodies; however, the same conditions produce immunoconjugates with a DOL of only ~1.5 when working with murine IgG1 antibodies. All this said, researchers should optimize these reaction conditions for new antibodies and PODS-bearing cargoes. Finally, and importantly, with respect to the final product, we have repeatedly and reproducibly found that PODS-based immunoconjugates exhibit immunoreactivities equal to or better than analogous constructs created using random or maleimide-based conjugation strategies.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic illustration of bioconjugations using (**A**) amine-reactive, (**B**) maleimide-bearing, and (**C**) PODS-bearing cargoes.

Figure 2. The Michael addition of a thiol-bearing biomolecule (green) and a radionuclide-bearing maleimide (yellow) to form a radiolabeled bioconjugate, as well as the additional reactions that the radiolabeled construct can undergo in the presence of endogenous thiol-bearing molecules (pink). RT = Room temperature. Figure reprinted with permission from Adumeau, P., Davydova, M., Zeglis, B. M. Thiol-Reactive Bifunctional Chelators for the Creation of Site-Selectively Modified Radioimmunoconjugates with Improved Stability. *Bioconjugate Chemistry*. **29**, 1364-1372 (2018). Copyright 2018 American Chemical Society.

Figure 3. Schematic of the reaction between PODS and a thiol. Figure reprinted with permission from Adumeau, P., Davydova, M., Zeglis, B. M. Thiol-Reactive Bifunctional Chelators for the Creation of Site-Selectively Modified Radioimmunoconjugates with Improved Stability. *Bioconjugate Chemistry*. **29**, 1364-1372 (2018). Copyright 2018 American Chemical Society.

Figure 4. The structure of PODS as well as the reagent reported by Barbas, et al.[18,19]

Figure 5 Scheme of the four-step synthesis of PODS. Figure reprinted with permission from Adumeau, P., Davydova, M., Zeglis, B. M. Thiol-Reactive Bifunctional Chelators for the Creation of Site-Selectively Modified Radioimmunoconjugates with Improved Stability. *Bioconjugate Chemistry*. **29**, 1364-1372 (2018). Copyright 2018 American Chemical Society.

Figure 6. Scheme of the synthesis of PODS-DOTA

Figure 7. Scheme of the bioconjugation of trastuzumab with PODS-DOTA.

Figure 8. Comparison of the in vivo behavior of ^{89}Zr-labeled radioimmunoconjugates of huA33 created using PODS-based (^{89}Zr-DFO-PODS-huA33) and maleimide-based (^{89}Zr-DFO-mal-huA33) bioconjugation strategies. Planar (left) and maximum intensity projection (right) PET images of athymic nude mice bearing A33 antigen-expressing SW1222 colorectal cancer xenografts (white arrow) following the injection of ^{89}Zr-DFO-PODS-huA33 and ^{89}Zr-DFO-mal-huA33 (140 μCi, 60-65 μg). The coronal slices intersect the center of the tumors. Figure reprinted with permission from Adumeau, P., Davydova, M., Zeglis, B. M. Thiol-Reactive Bifunctional Chelators for the Creation of Site-Selectively Modified Radioimmunoconjugates with Improved Stability. *Bioconjugate Chemistry*. **29**, 1364-1372 (2018). Copyright 2018 American Chemical Society.

Figure 9. Comparison of the in vivo behavior of ^{89}Zr-labeled radioimmunoconjugates of huA33 created using PODS-based (^{89}Zr-DFO-PODS-huA33) and maleimide-based (^{89}Zr-DFO-mal-huA33) bioconjugation strategies. Biodistribution data after the administration of ^{89}Zr-DFO-PODS-huA33 and ^{89}Zr-DFO-mal-huA33 (30 μCi, 15-18 μg) to athymic nude mice bearing A33 antigen-expressing subcutaneous SW1222 human colorectal cancer xenografts. The values for the stomach, small intestine, and large intestine include contents. Figure reprinted with permission from Adumeau, P., Davydova, M., Zeglis, B. M. Thiol-Reactive Bifunctional Chelators for the Creation of Site-Selectively Modified Radioimmunoconjugates with Improved Stability. *Bioconjugate Chemistry*. **29**, 1364-1372 (2018). Copyright 2018 American Chemical Society.

Table 1. Characterization data for the synthetic intermediates described as well as PODS and PODS-DOTA.

Table 2. Degree of labeling of different antibodies following conjugation with a PODS-bearing fluorophore. Values are shown ± standard deviations. Table reprinted with permission from Adumeau, P., Davydova, M., Zeglis, B. M. Thiol-Reactive Bifunctional Chelators for the Creation of Site-Selectively Modified Radioimmunoconjugates with Improved Stability. *Bioconjugate Chemistry*. **29**, 1364-1372 (2018). Copyright 2018 American Chemical Society.

DISCUSSION:

In this report, we have chosen not to include any protocols for radiolabeling or in vivo experimentation. Our reasons are straightforward. With respect to the former, the radiolabeling of a PODS-based immunoconjugate does not differ at all from that of an immunoconjugate synthesized using other bioconjugation strategies, and these procedures have been comprehensively reviewed elsewhere[2]. With regard to the latter, the specifics of preclinical in vivo experiments (i.e., mouse models, doses, etc.) can vary broadly according to both the application and the antibody/antigen system.

Our previous investigations with ^{89}Zr-labeled variants of huA33 provide a compelling illustration of the advantages of PODS-based bioconjugations. HuA33 is a humanized IgG1 antibody that targets the A33 antigen, a transmembrane glycoprotein expressed on >95% of colorectal cancers[23,24]. In our previous manuscript[22], we report the synthesis of ^{89}Zr-DFO-huA33 radioimmunoconjugate using both PODS- and maleimide-based bioconjugation strategies. The two radiolabeled antibodies — ^{89}Zr-DFO-PODS-huA33 and ^{89}Zr-DFO-mal-huA33 — were produced in nearly identical yield, purity, specificity activity, and immunoreactivity. Critically, however, the two radioimmunoconjugates exhibited dramatically different stabilities in human serum: after incubation for seven days at 37 °C, ^{89}Zr-DFO-PODS-huA33 remained 86 ± 1% intact, while its maleimide-based cousin was only 61 ± 5% intact. In vivo PET imaging and biodistribution experiments in athymic nude mice bearing A33 antigen-expressing SW1222 human colorectal cancer xenografts revealed stark differences in the in vivo behavior of the two radioimmunoconjugates (**Figure 8 and Figure 9**). Both ^{89}Zr-DFO-PODS-huA33 and ^{89}Zr-DFO-mal-huA33 produce high activity concentrations in tumor tissue: 56.4 ± 6.9%ID/g and 49.6 ± 9.3%ID/g, respectively, 48 h after administration. However, the maleimide-based radioimmunoconjugate produced significantly higher activity concentrations in healthy tissues than the PODS-based agent. For example, ^{89}Zr-DFO-mal-huA33 produced activity concentrations of 3.1 ± 0.5, 2.7 ± 0.4, and 12.2 ± 0.4% ID/g in the kidneys, liver, and bone, respectively, at 120 h post-injection, values which dramatically exceed the activity concentrations produced by ^{89}Zr-DFO-PODS-huA33 in the same tissues (1.4 ± 0.1, 1.2 ± 0.3, and 4.3 ± 0.6% ID/g). Indeed, ^{89}Zr-DFO-PODS-huA33 produced lower activity concentrations in all non-target tissues (except the large intestine) at 120 h post-injection compared to ^{89}Zr-DFO-mal-huA33. As a result, the tumor-to-organ activity concentration ratios for ^{89}Zr-DFO-PODS-huA33 are generally superior to those of ^{89}Zr-DFO-mal-huA33; in particular, the tumor-to-liver, tumor-to-spleen, tumor-to-kidney, and tumor-to-bone activity concentration ratios are nearly double for the PODS-based immunoconjugate compared to its maleimide-derived cousin.

Considering that the principal difference between the two radioimmunoconjugates was the bioconjugation handle of the chelator, the increased stability of the PODS-thiol linkage is almost certainly responsible for this improved in vivo performance.

Taking a broader view, the non-site-selective bioconjugation of probes to lysines within antibodies is admittedly a straightforward and facile approach to the modification of antibodies. However, the presence of multiple lysines distributed throughout the structure of immunoglobulins means that it is impossible to exert control over the precise site or degree of bioconjugation[2]. As a result, this random strategy often produces poorly-defined and highly heterogeneous immunoconjugates that can exhibit decreased immunoreactivity if ligations occur within the antigen-binding domains[3]. The benefits of site-selective approaches to bioconjugation have been illustrated repeatedly for both radioimmunoconjugates and antibody-drug conjugates[8,14,25-30]. In short, not only do site-selective bioconjugation strategies produce more well-defined and homogenous immunoconjugates than traditional methodologies, they also create imaging agents, radioimmunotherapeutics, and ADCs with improved in vivo performance. Yet where do PODS-based ligations stand in comparison to other site-selective modification strategies? Generally speaking, the approaches to the site-selective modification of antibodies can be classified into four categories: (1) ligations to cysteine residues, (2) the manipulation of the heavy chain glycans, (3) chemoenzymatic transformations, and (4) the use of genetic engineering[4,5]. Of course, this classification system is not perfect, and some approaches (e.g., the modification of the heavy chain glycans with enzymes) inevitably qualify for two categories. Each strategy has its own advantages and disadvantages. Genetic engineering-based approaches provide exquisite control over the site of conjugation, yet they are complex and expensive[31-33]. Oxidative couplings to the heavy chain glycans are inexpensive and straightforward, yet they risk oxidative damage to the structural integrity of the immunoglobulin[34-38].

The chief advantage of thiol-based bioconjugations — PODS included — is their simplicity and modularity. Their principal limitation, on the other hand, stems from the presence of multiple thiols within an antibody, a trait which reduces the degree of control over both the site of conjugation and the number of modifications per antibody. In this sense, the combination of thiol-based ligations and antibodies that have been genetically engineered to possess free cysteine residues is a particularly attractive approach. As we have noted, another limitation of maleimide-based thiol ligations is the susceptibility of the succinimidyl thioether bond to retro-Michael additions in vivo. Yet critically, the use of PODS abrogates this problem.

Before we conclude, it is important to note that the emergent nature of PODS technology can create its own set of obstacles. For example, no PODS-bearing bifunctional chelators are (currently) commercially available, and there is no data addressing the clinical pharmacology, toxicology, or immunogenicity of PODS-based immunoconjugates. However, we believe that PODS-based bioconjugations have the potential to fundamentally change the way immunoconjugates are synthesized in both the laboratory and clinic. At present, we have only applied this chemical technology to the development of radioimmunoconjugates for nuclear imaging and radioimmunotherapy, though investigations into the utility of this approach for the

construction of antibody-drug conjugates and other biomolecular medicines are currently underway. In the end, we earnestly hope that this protocol — and particularly the straightforward and simple chemistry that we have developed — will help promote the use of phenyloxadiazolyl methyl sulfones for sulfhydryl-based conjugations and spur a shift in the field from maleimides to more stable and more reliable alternatives.

ACKNOWLEDGMENTS:

The authors thank Dr. Sai Kiran Sharma for helpful conversations.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Wu, A. M. Antibodies and antimatter: The resurgence of immuno-PET. *Journal of Nuclear Medicine.* **50** (1), 2-5 (2009).

2 Zeglis, B. M., Lewis, J. S. A practical guide to the construction of radiometallated bioconjugates for positron emission tomography. *Dalton Transactions.* **40** (23), 6168-6195 (2011).

3 Agarwal, P., Bertozzi, C. R. Site-specific antibody-drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. *Bioconjugate Chemistry.* **26** (2), 176-192 (2015).

4 Adumeau, P., Sharma, S. K., Brent, C., Zeglis, B. M. Site-specifically labeled immunoconjugates for molecular imaging-part 1: Cysteine residues and glycans. *Molecular Imaging and Biology.* **18** (1), 1-17 (2016).

5 Adumeau, P., Sharma, S. K., Brent, C., Zeglis, B. M. Site-specifically labeled immunoconjugates for molecular imaging-part 2: Peptide tags and unnatural amino acids. *Molecular Imaging and Biology.* **18** (1), 153-165 (2016).

6 Alley, S. C. et al. Contribution of linker stability to the activities of anticancer immunoconjugates. *Bioconjugate Chemistry.* **19** (3), 759-765 (2008).

7 Baldwin, A. D., Kiick, K. L. Tunable degradation of maleimide-thiol adducts in reducing environments. *Bioconjugate Chemistry.* **22** (10), 1946-1953 (2011).

8 Shen, B.-Q. et al. Conjugation site modulates the *in vivo* stability and therapeutic activity of antibody-drug conjugates. *Nature Biotechnology.* **30** (2), 184-189 (2012).

9 Jackson, D. et al. In vitro and *in vivo* evaluation of cysteine and site specific conjugated herceptin antibody-drug conjugates. *Plos One.* **9** (1) (2014).

10 Ponte, J. F. et al. Understanding how the stability of the thiol-maleimide linkage impacts the pharmacokinetics of lysine-linked antibody-maytansinoid conjugates. *Bioconjugate Chemistry.* **27** (7), 1588-1598 (2016).

11 Stimmel, J. B. et al. Site-specific conjugation on serine -> cysteine variant monoclonal antibodies. *Journal of Biological Chemistry.* **275** (39), 30445-30450 (2000).

12 Li, L. et al. Reduction of kidney uptake in radiometal labeled peptide linkers conjugated to recombinant antibody fragments. site-specific conjugation of DOTA-peptides to a cys-diabody. *Bioconjugate Chemistry.* **13** (5), 985-995 (2002).

13 Li, J., Wang, X. H., Wang, X. M., Chen, Z. L. Site-specific conjugation of bifunctional chelator BAT to mouse IgG(1) Fab' fragment. *Acta Pharmacologica Sinica.* **27** (2), 237-241 (2006).
14 Tinianow, J. N. et al. Site-specifically Zr-89-labeled monoclonal antibodies for ImmunoPET. *Nuclear Medicine and Biology.* **37** (3), 289-297 (2010).
15 Li, L. et al. Site-specific conjugation of monodispersed DOTA-PEGn to a thiolated diabody reveals the effect of increasing PEG size on kidney clearance and tumor uptake with improved 64-copper PET imaging. *Bioconjugate Chemistry.* **22** (4), 709-716 (2011).
16 Khalili, H., Godwin, A., Choi, J.-w., Lever, R., Brocchini, S. Comparative binding of disulfide-bridged PEG-Fabs. *Bioconjugate Chemistry.* **23** (11), 2262-2277 (2012).
17 Koniev, O., Wagner, A. Developments and recent advancements in the field of endogenous amino acid selective bond forming reactions for bioconjugation. *Chemical Society Reviews.* **44** (15), 5495-5551 (2015).
18 Patterson, J. T., Asano, S., Li, X., Rader, C., Barbas, C. F., III. Improving the serum stability of site-specific antibody conjugates with sulfone linkers. *Bioconjugate Chemistry.* **25** (8), 1402-1407 (2014).
19 Toda, N., Asano, S., Barbas, C. F., III. Rapid, stable, chemoselective labeling of thiols with Julia-Kocienski-like reagents: A serum-stable alternative to maleimide-based protein conjugation. *Angewandte Chemie-International Edition.* **52** (48), 12592-12596 (2013).
20 Zhang, Q. et al. Last-step enzymatic F-18-fluorination of cysteine-tethered RGD peptides using modified Barbas linkers. *Chemistry-a European Journal.* **22** (31), 10998-11004 (2016).
21 Chiotellis, A. et al. Novel chemoselective F-18-radiolabeling of thiol-containing biomolecules under mild aqueous conditions. *Chemical Communications.* **52** (36), 6083-6086 (2016).
22 Adumeau, P., Davydova, M., Zeglis, B. M. Thiol-reactive bifunctional chelators for the creation of site-selectively modified radioimmunoconjugates with improved stability. *Bioconjugate Chemistry.* **29** 1364-1372 (2018).
23 Sakamoto, J., Kojima, H., Kato, J., Hamashima, H., Suzuki, H. Organ-specific expression of the intestinal epithelium-related antigen A33, a cell surface target for antibody-based imaging and treatment in gastrointestinal cancer. *Cancer Chemotherapy and Pharmacology.* **46** S27-S32 (2000).
24 Sakamoto, J. et al. A phase I radioimmunolocalization trial of humanized monoclonal antibody huA33 in patients with gastric carcinoma. *Cancer Science.* **97** (11), 1248-1254 (2006).
25 Junutula, J. R. et al. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nature Biotechnology.* **26** (8), 925-932 (2008).
26 Pillow, T. H. et al. Site-specific trastuzumab maytansinoid antibody-drug conjugates with improved therapeutic activity through linker and antibody engineering. *Journal of Medicinal Chemistry.* **57** (19), 7890-7899 (2014).
27 Boswell, C. A. et al. Enhanced tumor retention of a radiohalogen label for site-specific modification of antibodies. *Journal of Medicinal Chemistry.* **56** (23), 9418-9426 (2013).
28 Boswell, C. A. et al. Impact of drug conjugation on pharmacokinetics and tissue distribution of anti-STEAP1 antibody-drug conjugates in rats. *Bioconjugate Chemistry.* **22** (10), 1994-2004 (2011).

29 Alvarez, V. L. et al. Site-specifically modified In-111 labeled antibodies give low liver backgrounds and improved radioimmunoscintigraphy. *Nuclear Medicine and Biology.* **13** (4), 347-352 (1986).
30 Strop, P. et al. Location matters: SIte of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. *Chemistry, Biology.* **20** (2), 161-167 (2013).
31 Hallam, T. J., Wold, E., Wahl, A., Smider, V. V. Antibody conjugates with unnatural amino acids. *Molecular Pharmaceutics.* **12** (6), 1848-1862 (2015).
32 Axup, J. Y. et al. Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proceedings of the National Academy of Sciences.* **109** (40), 16101-16106 (2012).
33 Lang, K., Chin, J. W. Cellular incorporation of unnatural amino acids and bioorthogonal labeling of proteins. *Chemical Reviews.* **114** (9), 4764-4806 (2014).
34 Yamasaki, R. B., Osuga, D. T., Feeney, R. E. Periodate oxidation of methionine in proteines. *Analytical Biochemistry.* **126** (1), 183-189 (1982).
35 Wang, W. et al. Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies. *Molecular Immunology.* **48** (6-7), 860-866 (2011).
36 O'Shannessy, D. J., Dobersen, M. J., Quarles, R. H. A novel procedure for labeling immunoglobulins by conjugation to oligosaccharide moieties. *Immunology Letters.* **8** (5), 273-277 (1984).
37 Panowski, S., Bhakta, S., Raab, H., Polakis, P., Junutula, J. R. Site-specific antibody drug conjugates for cancer therapy. *Mabs.* **6** (1), 34-45 (2014).
38 Hu, M. D. et al. Site-specific conjugation of HIV-1 tat peptides to IgG: a potential route to construct radioimmunoconjugates for targeting intracellular and nuclear epitopes in cancer. *European Journal of Nuclear Medicine and Molecular Imaging.* **33** (3), 301-310 (2006).

Figure 1

Click here to access/download;Figure;Figure1.tif

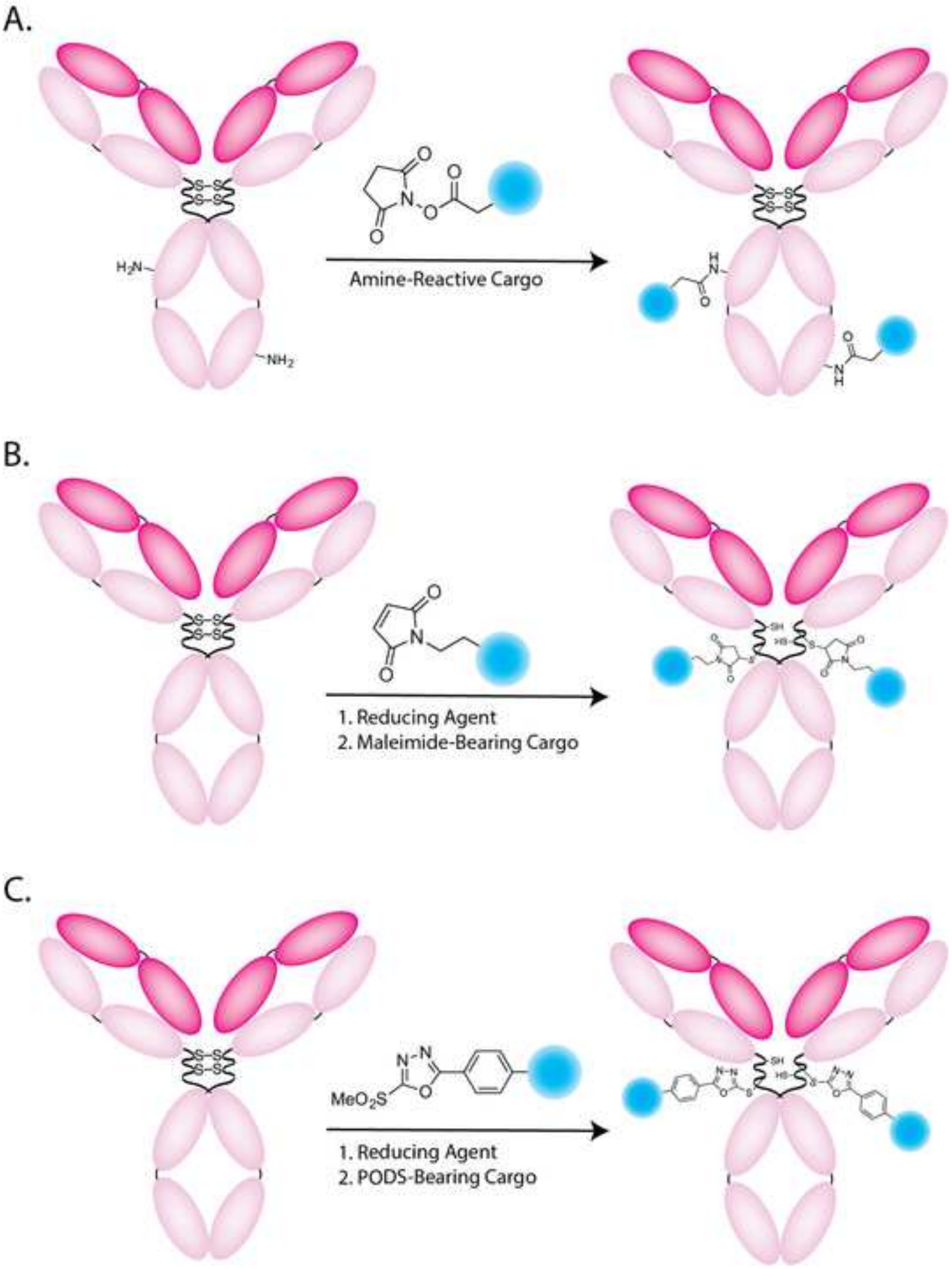

Figure 2

Click here to access/download;Figure;Figure2_Revised.tif

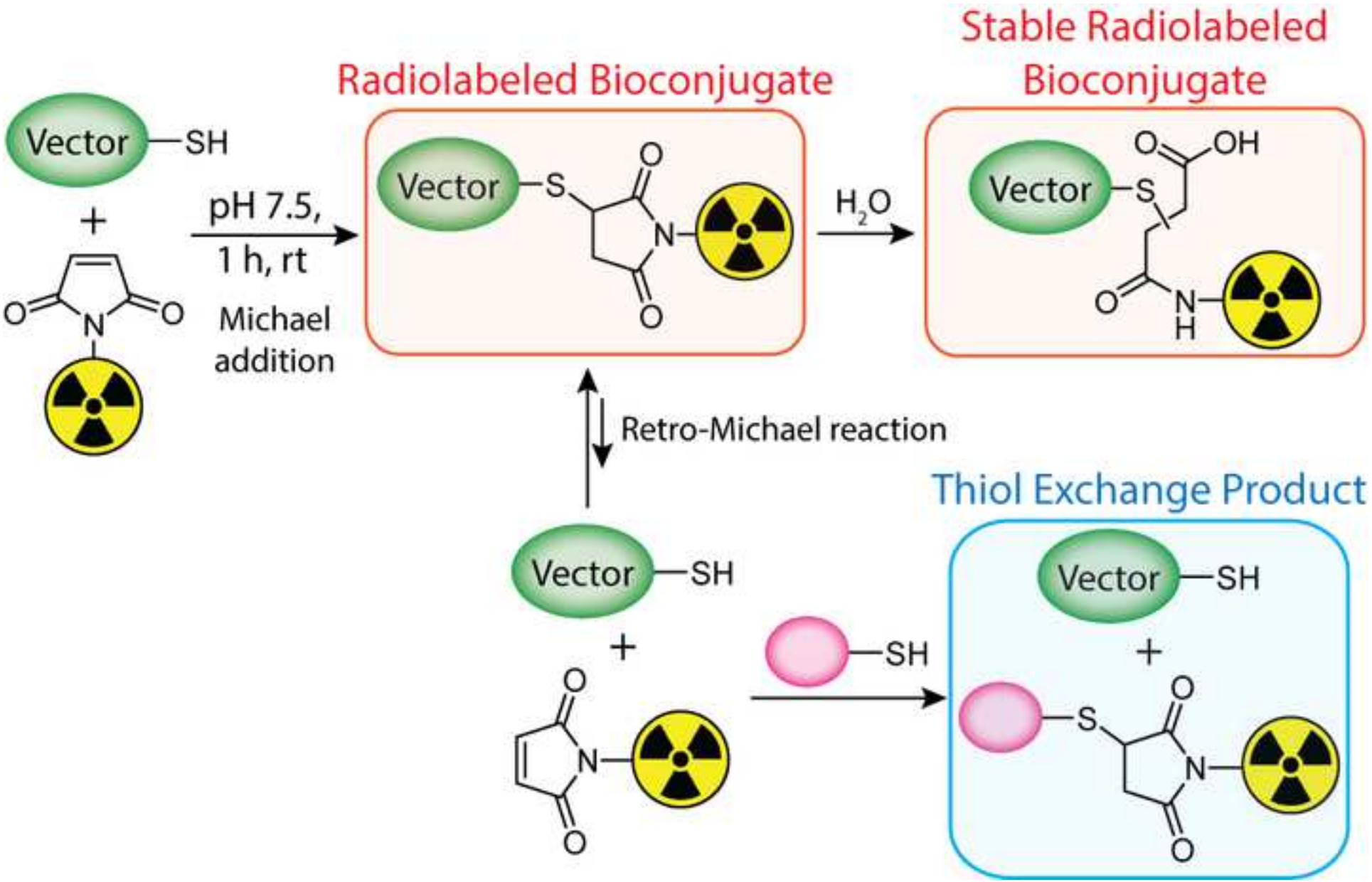

Figure 3

Click here to access/download;Figure;Figure3.tif

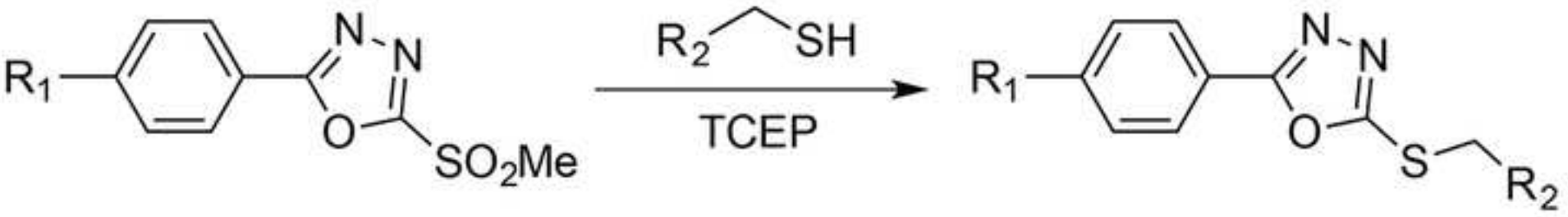

Figure 4

Click here to access/download;Figure;Figure4_New.tif

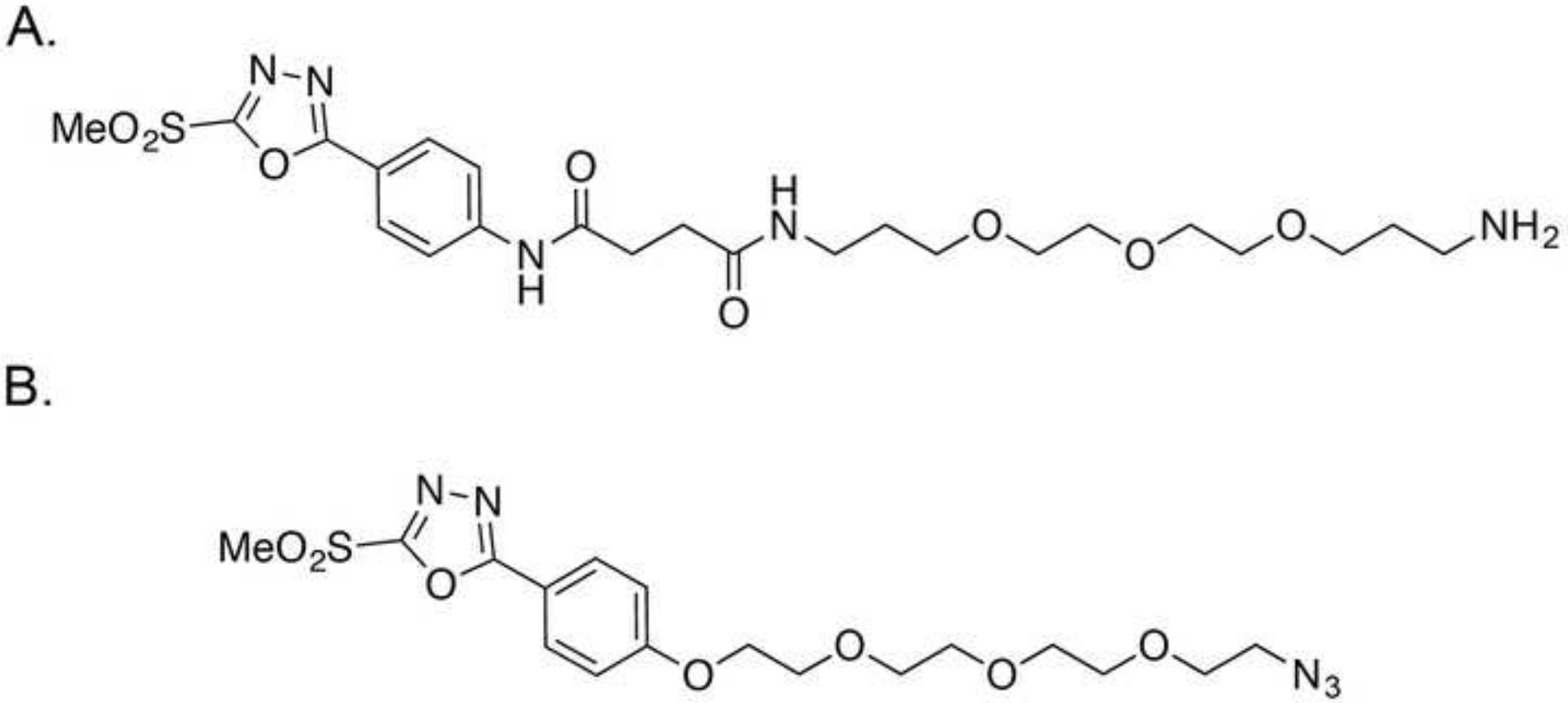

Figure 5

Click here to access/download;Figure;Figure5_Revised.tif

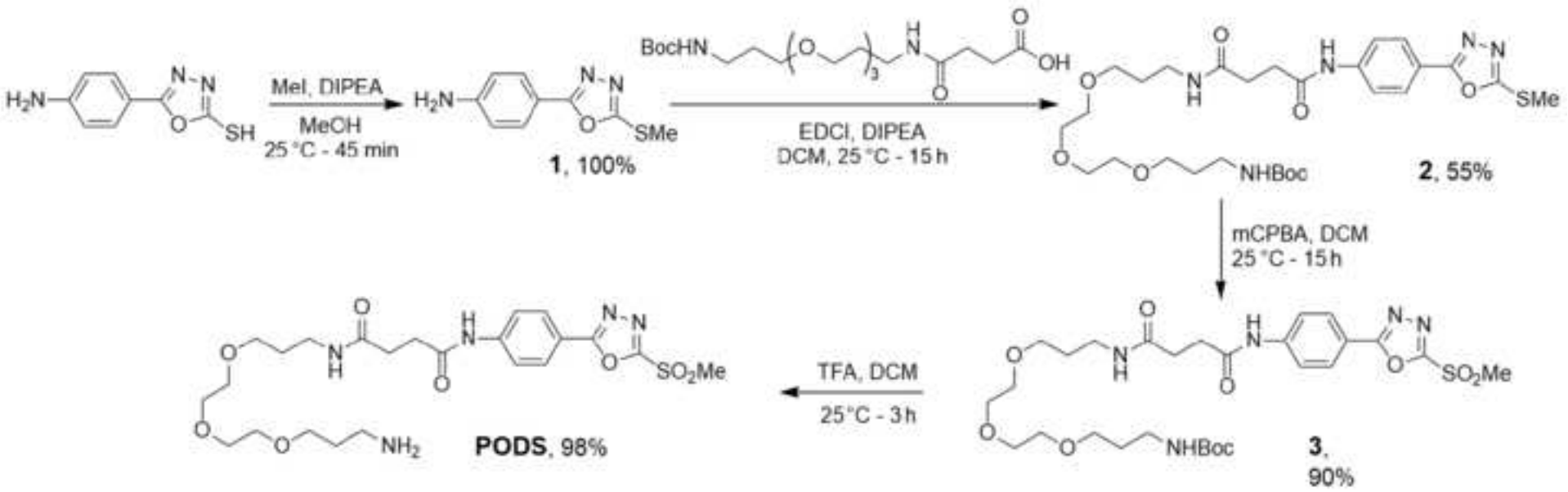

Figure 6

Click here to access/download;Figure;Figure6_Revised.tif

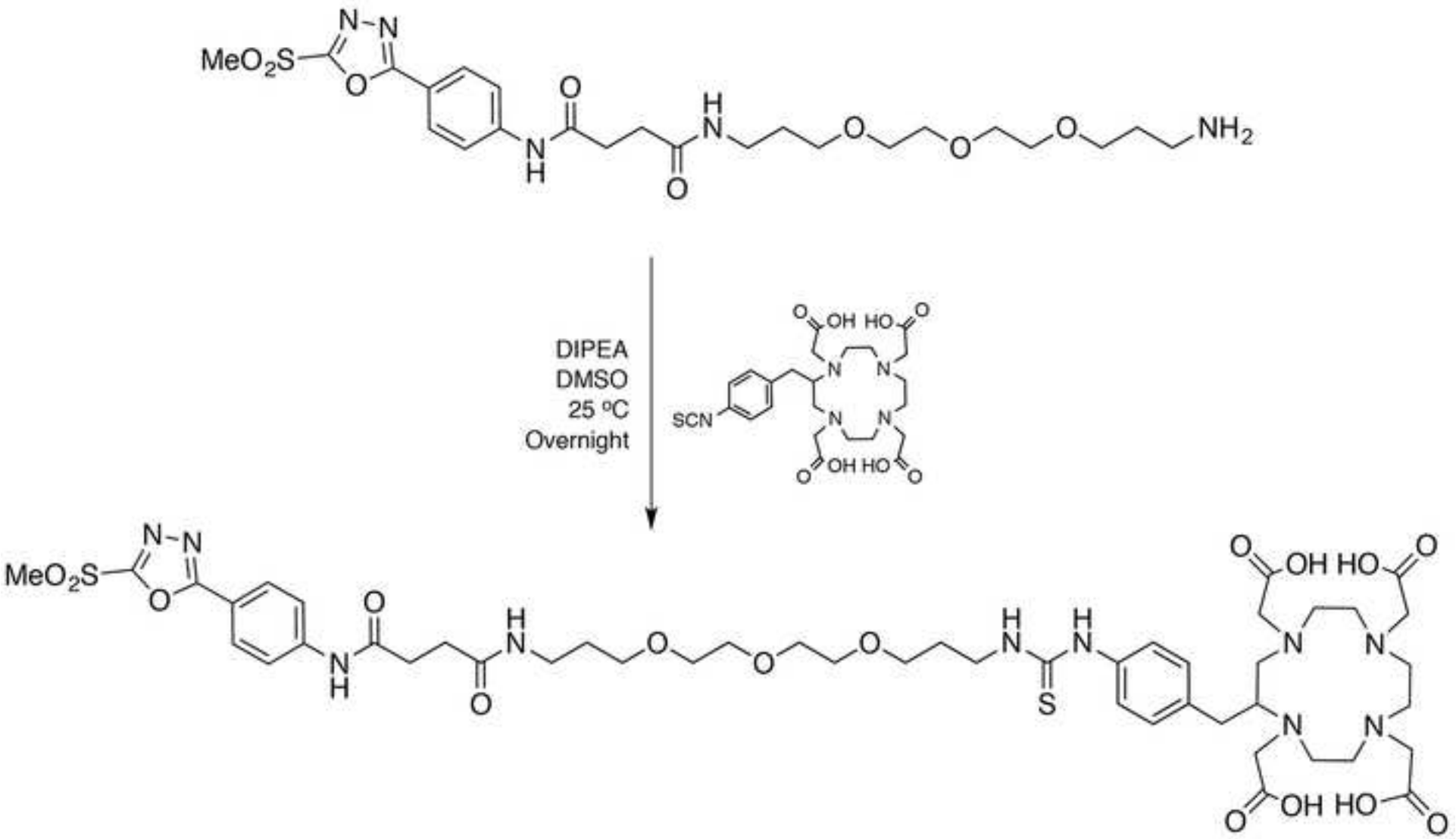

Figure 7

Click here to access/download;Figure;Figure7_Revised.tif

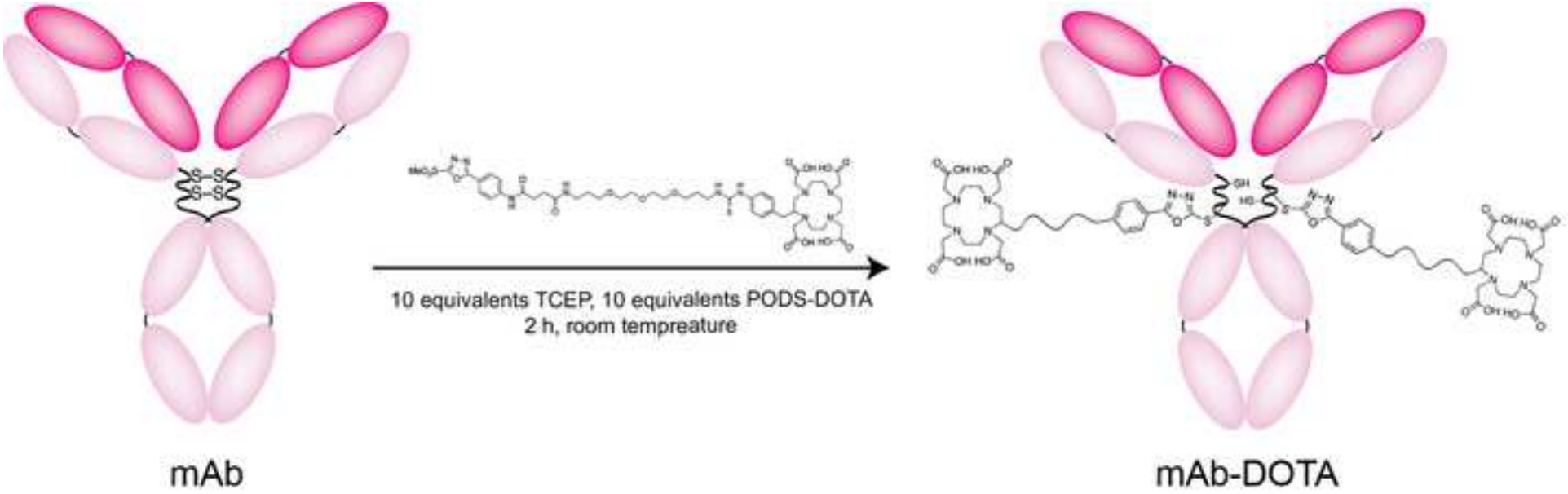

Figure 8

Click here to access/download;Figure;Figure8.tif

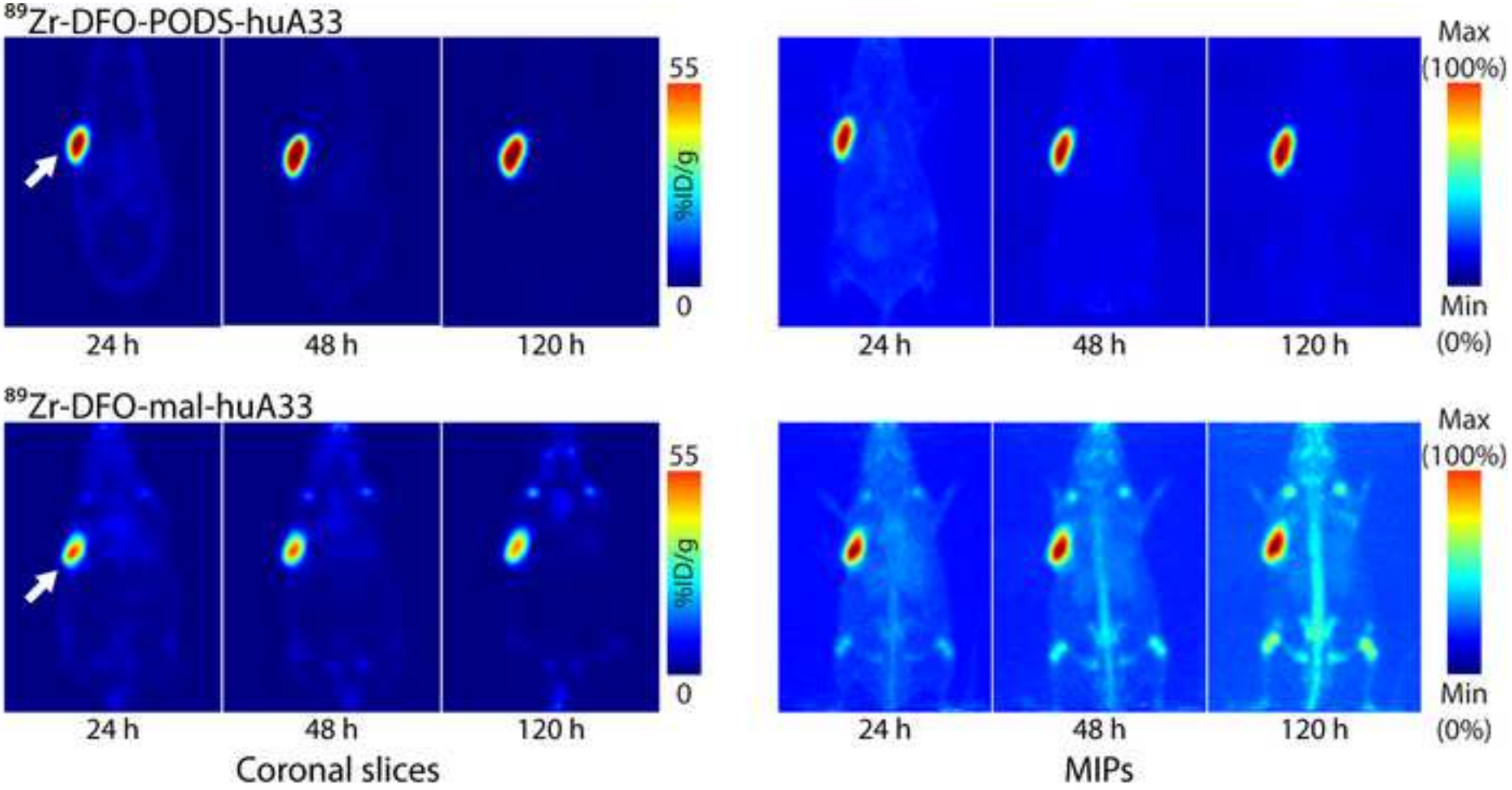

Figure 9

Click here to access/download;Figure;Figure9.tif

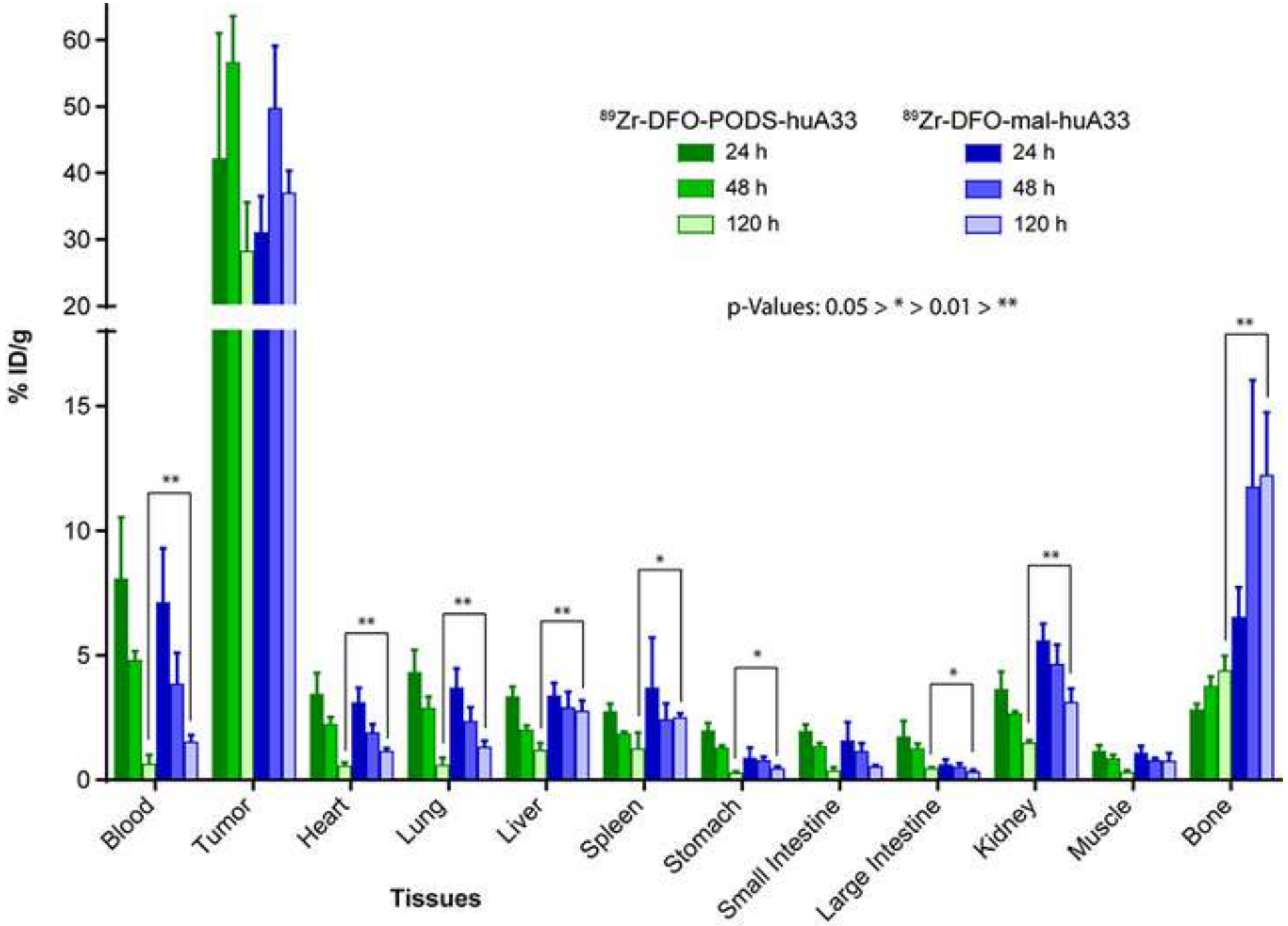

Table 1 Click here to access/download;Table;Revised_Table1.xlsx

Compound	^{1}H-NMR Shifts	^{13}C-NMR Shifts
1	(500 MHz, $CDCl_3$) 7.79 (2H, d, J = 8.5 Hz), 6.72 (2H, d, J = 8.5 Hz), 4.04 (2H, br s), 2.75 (3H, s)	(125 MHz, $CDCl_3$) 166.3, 163.7, 149.7, 128.5, 114.8, 113.5, 14.8
2	(500 MHz, CDCl3) 9.68 (1H, s), 7.91 (2H, d, J = 9.0 Hz), 7.71 (2H, d, J = 8.5 Hz), 6.82 (1H, s), 4.99 (1H, s), 3.70-3.45 (12H, m), 3.41 (2H, q, J = 6.0 Hz), 3.20 (2H, q, J = 6.5 Hz), 2.76 (3H, s), 2.71 (2H, m), 2.63 (2H, m), 1.80-1.70 (4H, m), 1.42 (9H, s)	(125 MHz, CDCl3) 172.6, 171.3, 165.8, 164.6, 156.2, 141.8, 127.7, 119.6, 118.6, 79.2, 70.6, 70.5, 70.3, 70.1, 69.6, 38.8, 38.5, 33.5, 31.6, 29.9, 28.6, 14.8
3	(500 MHz, $CDCl_3$) 9.99 (1H, s), 7.98 (2H, d, J = 9.0 Hz), 7.75 (2H, d, J = 8.5 Hz), 6.88 (1H, s), 4.99 (1H, s), 3.66-3.50 (15H, m), 3.41 (2H, q, J = 6.0 Hz), 3.20 (2H, q, J = 6.5 Hz), 2.71 (2H, m), 2.65 (2H, m), 1.80-1.70 (4H, m), 1.43 (9H, s)	(125 MHz, $CDCl_3$) 172.6, 171.5, 166.5, 161.6, 156.1, 143.4, 128.7, 119.6, 116.4, 79.1, 70.5, 70.4, 70.2, 70.0, 69.4, 43.0, 38.8, 38.4, 33.2, 31.3, 29.7, 28.4

PODS	(500 MHz, D_2O) 7.85 (2H, d, J = 9.0 Hz), 7.55 (2H, d, J = 8.5 Hz), 3.60-3.45 (15H, m), 3.45 (2H, t, J = 6.5 Hz), 3.20 (2H, t, J = 6.5 Hz), 3.04 (2H, t, J = 7.0 Hz), 2.67 (2H, t, J = 6.5 Hz), 2.54 (2H, t, J = 6.5 Hz), 1.87 (2H, qt, J = 6.5 Hz), 1.70 (2H, qt, J = 6.5 Hz)	(125 MHz, D_2O) 174.5, 173.2, 166.8, 161.4, 142.2, 128.6, 120.3, 116.6, 69.4, 69.4, 69.3, 69.2, 68.2, 68.2, 42.5, 37.6, 36.2, 31.9, 30.7, 28.2, 26.4
PODS-DOTA	(600 MHz, DMSO-d_6) 10.46 (1H, s), 9.74 (1H, bs), 8.04 (2H, d, J = 8.6 Hz), 7.99 (1H, s), 7.90 (1H, t, J = 5.0 Hz), 7.86 (2H, d, J = 6.5 Hz), 7.44 (2H, d, J = 7.9 Hz), 7.24 (2H, d, J = 7.1 Hz), 4.35-2.41 (45H, m), 3.70 (3H, s), 1.76 (2H, q, J = 6.3 Hz), 1.61 (2H, q, J = 6.5 Hz)	(125 MHz, DMSO-d_6) 171.8, 171.4, 166.1, 162.2, 158.8, 158.6, 129.8, 129.0, 127.6, 123.3, 119.5, 118.5, 116.5, 116.4, 70.2, 70.1, 70.0, 68.7, 68.5, 43.4, 41.8, 36.3, 32.2, 30.4, 29.8, 29.1

HRMS
m/z Calcd for $[C_9H_9N_3OS+H]^+$: 208.0539; found: 208.0539; Δ: 0.0 ppm
m/z Calcd for $[C_{28}H_{43}N_5O_8S+Na]^+$: 632.2725; found: 632.2722; Δ: -0.47 ppm
m/z Calcd for $[C_{28}H_{43}N_5O_{10}S+H]^+$: 642.2803; found: 642.2797; Δ: -0.93 ppm

m/z Calcd for $[C_{23}H_{35}N_5O_8S+H]^+$: 542.2279; found: 542.2281; Δ: 0.37 ppm
m/z Calcd for $[C_{47}H_{68}N_{10}O_{16}S_2+H]^+$: 1093.4334; found: 1093.4327; Δ: -0.64 ppm

Table 2

Click here to access/download;Table;Revised_Table2.xlsx

Antibody	Type	Constant Region	Ratio of PODS:mAb
Human plasma IgG	Human	Human IgG	2.1 ± 0.1
Trastuzumab	Humanized	Human IgG1	2.0 ± 0.1
huA33	Humanized	Human IgG1	2.1 ± 0.1
Cetuximab	Chimeric	Human IgG1	2.2 ± 0.1
AR 9.6	Murine	Murine IgG1	1.4 ± 0.1
Mouse plasma IgG	Murine	Murine IgG	1.5 ± 0.1

Table of Materials

Click here to access/download;Table of Materials;Revised_TableOfMaterials.xlsx

Name	Company	Catalog Number
5-(4-aminophenyl)-1,3,4-oxadiazole-2-thiol	Sigma-Aldrich	675024
1.5 mL LoBind Microcentrifugal Tube	Eppendorf	925000090
1.5 mL Microcentrifugal Tube	Fisherbrand	05-408-129
Acetonitrile	Fisher Scientific	A998-4
Amicon Ultra-2 Centrifugal Filter Unit	EMD Millipore	EN300000141G
Cyclohexane	Fisher Scientific	C556-4
Dichloromethane	Fisher Scientific	AC383780010
Diisopropylethylamine	MP Biomedicals, LLC	150915
Dimethylsulfoxide	Fisher Scientific	31-727-5100ML
Ethyl Acetate	Fisher Scientific	E145 4
Hydrochloric Acid	Fisher Scientific	A144-500
Iodomethane	Sigma-Aldrich	289566-100G
Magnesium Sulfate	Acros Organics	413485000
m-chloroperbenzoic acid	Sigma-Aldrich	273031
Methanol	Fisher Scientific	A412 1
NBoc-N'-succinyl-4,7,10-trioxa-1,13-tridecanediamine	Sigma-Aldrich	671401
N-ethyl-N'- [3-(dimethylamino)propyl] carbodiimide hydrochloride	Sigma-Aldrich	3450
Phosphate Buffered Saline	Sigma-Aldrich	P5493
p-SCN-Bn-DOTA	Macrocyclics	B-205
Sephadex G-25 in PD-10	GE Healthcare	17085101
Sodium Carbonate	Sigma-Aldrich	S7795
Sodium Hydroxide	Fisher Scientific	S318-1
TCEP	ThermoFischer Scientific	20490
Triethylamine	Fisher Scientific	AC157911000
Trifluoroacetic Acid	Fisher Scientific	A116-50

Comments
Store at -80 °C
10× Concentration
Store at -80 °C

Author License Agreement (ALA)
Click here to access/download;Author License Agreement (ALA);JoVE_PODS_LicenseAgreement.pdf

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Synthesis and Bioconjugation of Thiol-Reactive Reagents for the Creation of Site-Selectively Modified Immunoconjugates
Author(s):	Maria Davydova, Guillaume Dewaele Le Roi, Pierre Adumeau, Brian Zeglis

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

[X] Standard Access [ ] Open Access

Item 2: Please select one of the following items:

[X] The Author is **NOT** a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Brian M. Zeglis
Department:	Chemistry
Institution:	Hunter College of the City University of New York
Title:	Assistant Professor
Signature:	
Date:	September 5th, 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Rebuttal Letter
Click here to access/download;Rebuttal Letter;JoVE_PODS_ResponseToReviewers_10-31-2018.pdf

Brian M. Zeglis, Ph.D.
Assistant Professor, Department of Chemistry
Hunter College of the City University of New York
Assistant Affiliate Member, Department of Radiology
Memorial Sloan Kettering Cancer Center
Belfer Research Building, 413 East 69th Street, New York, NY 10021

October 31th, 2018

Phillip Steindel, Ph.D.
Review Editor, *JoVE*

Dear Dr. Steindel,

Attached please find our revisions to the manuscript we have submitted to *JoVE* entitled "Synthesis and Bioconjugation of Thiol-Reactive reagents for the Creation of Site-Specifically Modified Immunoconjugates" (JoVE59063). We would like to thank you and the reviewers for your consideration of our work and your careful review of the manuscript. We have made a number of critical modifications to the manuscript based on the critiques offered in the review. As requested, we have addressed these changes on a point-by-point basis. Below we have listed the comments of the Reviewers 1, 2, and 3 (in **bold**) along with our responses to said comments and the changes we have made (in normal text). For the sake of clarity, any addition or modification to the text in the manuscript are listed below in *purple italics*.

Editorial Comments:

"Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues."

Thank you! We have now thoroughly proof-read the manuscript.

"Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."""

All of the figures in this manuscript are either original or have been modified from our own work that was recently published in *Bioconjugate Chemistry*. Therefore, according to the rules of the American Chemical Society, permissions are not required. However, we have added the appropriate references to the original manuscript where appropriate: *"Figure reprinted with permission from Adumeau, P., Davydova, M., Zeglis, B. M. Thiol-Reactive Bifunctional Chelators for the Creation of Site-Selectively Modified Radioimmunoconjugates with Improved Stability. Bioconjugate Chemistry.* ***29****, 1364-1372, (2018). Copyright 2018 American Chemical Society."*

"Please shorten the Summary to no more than 50 words."

The summary has now been shortened to 49 words: *"The in vivo performance of radiolabeled immunoconjugates for nuclear imaging and radioimmunotherapy is dependent on the stable bioconjugation of the radionuclide to the immunoglobulin. In this protocol, we will describe the synthesis of PODS, a phenyoxadiazolyl methyl sulfone-based reagent for the site-specific attachment of cargos to the thiols of biomolecules, particularly antibodies. In addition, we will describe the synthesis and characterization of a PODS-bearing bifunctional chelator and its conjugation to a model*

antibody."

"Please rephrase the Introduction to include a clear statement of the overall goal of this method."

We have now added the following sentence to make the goal clear, *"The over-arching goal of our development of PODS is to facilitate the creation of well-defined, homogeneous, highly stable, and highly immunoreactive immunoconjugates for in vitro and in vivo applications."*

"JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sephadex, GE Healthcare, Millipore Corp., AmiconTM, etc."

All commercial language has been removed from the manuscript.

"Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below."

We have added many more details to the protocol in the manuscript. Please see our responses to **Reviewer #2** for the exact edits.

"1.4: This is not clear. Is a vacuum dryer used?"

This has been revised. It now reads, "*1.4* Using a rotary evaporator, set the water bath to 40°C and slowly reduce the pressure to remove the solvent to afford a white solid."

"1.5: What volume of sodium carbonate is used to wash? Please specify how to perform the wash step."

This has been revised. It now reads, *"1.5 Dissolve the solid in 3 mL of ethyl acetate and wash at least three times with a 5 mL solution of 0.1 M sodium carbonate using a separatory funnel."*

"1.7: Is magnesium sulfate added to the organic phase? What amount is added?"

This has been revised. It now reads, *"1.7 Collect the organic phase and add magnesium sulfate to remove any traces of water. Note: The magnesium sulfate should be added with a small spatula and the solution should be swirled. If fine particles of the drying agent are still seen, the solution is dry. If not, add another small amount of the magnesium sulfate."*

"6.5: Please describe how to purify via size exclusion chromatography."

This has been revised. It now reads:

"6.5 After 2 hours, purify the immunoconjugate using a pre-packed disposable size exclusion desalting column.

6.5.1 First, equilibrate the size exclusion column as described by the supplier to remove any preservatives present in the column during storage. A typical procedure involves washing the column 5 times with a volume of PBS that corresponds to the volume of the column: 5 x 2.5 mL of PBS.

6.5.2 Next, add the reaction mixture to the size exclusion column noting the volume of the reaction mixture.

6.5.3 After the reaction mixture has entered the column, add an appropriate amount of PBS to bring the total volume of solution added to the column up to 2.5 mL. For example, if the conjugation reaction resulted in a total volume of 1.3 mL, 1.2 mL of additional PBS would need to be added to the column.

6.5.4 Finally, collect the product using 2 mL of PBS as the eluent."

"6.6: Please specify the molecular weight cut-off of the filtration unit."

This has been revised. It now reads, *"6.6 Concentrate the final immunoconjugate with centrifugal filtration units with a 50 kDa molecular weight cut-off."*

"After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please note that some of the shorter Protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step."

We have highlighted the relevant sections.

"Figure 2 and Figure 4: Please insert a space between numbers and their corresponding units (1 h, 3 h, 45 min, 25 °C). Please define "rt" in the figure legend."

These edits have been made to Figures 2 and 4. The legend for Figure 2 now reads, *"rt = room temperature".*

"Table 2: Please insert a space before and after the "±" symbol (i.e., 2.1 ± 0.1). Please also explain what the numbers after the "±" symbol represent."

These edits have been made to Table 2. The legend for Table 2 now reads, *"Values are shown ± standard deviations."*

"JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: (a) Critical steps within the protocol; (b) Any modifications and troubleshooting of the technique; (c) Any limitations of the technique; (d) The significance with respect to existing methods; (e) Any future applications of the technique."

We have intentionally designed the synthetic scheme and the bioconjugation strategy to be simple, and thus, there are very few critical steps or points for troubleshooting within the protocol. That said, in the "Representative Results", we have addressed a couple of key areas in which the protocol may need to be amended (underlining added for emphasis), *"This result remains consistent across a range of human, humanized, and chimeric IgG1 antibodies; however, the same conditions produce immunoconjugates with a DOL of only ~1.5 when working with murine IgG1 antibodies. All this said, researchers should optimize these reaction conditions for new antibodies and PODS-bearing cargoes."*

At bottom, while it is no doubt important to address *how* things are done in this protocol — and we believe we have done so ably — we believe it is just as important to describe *why* readers should adopt this new technology over chemical tools (maleimides) that the field has used for decades. In light of this, the second paragraph of the "Discussion" is dedicated to a clear, data-driven description of how PODS-based radioimmunoconjugates are superior to maleimide-based radioimmunoconjugates. This is *critical* to the manuscript as whole. The third and fourth paragraphs of the "Discussion" aim to place PODS-based ligations within the scientific context of site-specific bioconjugations as a whole and explain the relative advantages of thiol-based — and specificity PODS-based — bioconjugations. Finally, the fifth paragraph of the "Discussion" clearly states some of the potential drawbacks of PODS-based bioconjugations.

Admittedly, we had not addressed any possible future directions of research. As a result, we have now added the

Phone: 212.896.0443 e-mail: brian.zeglis@hunter.cuny.edu

following passage to the "Discussion": *"At present, we have only applied this chemical technology to the development of radioimmunoconjugates for nuclear imaging and radioimmunotherapy, though investigations into the utility of this approach to the construction of antibody-drug conjugates and other biomolecular medicines are currently underway."* With this addition, we sincerely believe we have covered all of the points that you wish us to address in the "Discussion" while also adding some "*why*" information for readers along the way.

"Please revise the table of the essential supplies, reagents, and equipment to include the name, company, and catalog number of all relevant materials/equipment."

The Table of Materials has been revised accordingly.

Reviewer #1:

"In this manuscript the authors provide a useful and very well described protocol … The interest of the method lies in its modularity (other cargos can be appended to PODS) and in the improved stability of the link between PODS and thiols groups when compared to the classical approach using maleimide compounds. This was demonstrated in a previous work reported by the authors (Bioconjugate Chem 2018) involving a PODS-DFO bifunctional chelating agent for 89Zr radioimmunoPET. Some results of this previous study (in vivo imaging, biodistribution) are presented in the manuscript. The radiometallation of the conjugate and in vivo studies are not reported in this manuscript because these steps are not different from those used for any other conjugates obtained by classical bioconjugation routes."

We thank the reviewer for their thorough and thoughtful review of our work.

"Lines 89 to 108. The name (phenyloxadiazolylmethyl sulfone) and the structure (Figures 1C and 3) of PODS reported in the manuscript (lines 107 & 108) are exactly the same as the one reported by Barbas (lines 90 & 91). It is said in the paragraph (lines 89 to 103) that the commercially available reagent was not pure enough, and that the synthesis of this reagent was not a realistic option. But, in the following paragraph, it appears that the synthesis of the same compound was actually the purpose of the work. This point has to be clarified."

This is an excellent point! The different between the reagent published by Barbas and the PODS reagent is that the former is based on a phenol ring while the latter is based on an aniline ring. This small change makes the synthesis of the molecule much more accessible and facile and makes the molecule itself more stable. In order to clarify this point, we have added the following sentences to the introduction, *"The key difference between PODS and the reagent reported by Barbas, et al. is that the former employs an aniline ring attached to the phenyloxadiazolyl methyl sulfone moiety, while the latter features a phenol in the same position. This change facilitates a more straightforward and accessible synthetic route as well as — if our experience with the commercially available reagent is emblematic — a more stable final reagent."* In addition, we have added a new figure — Figure 4 — that makes the distinction more clear.

"In the structure of compounds 2, 3 and PODS in figure 4, there are 3 carbon atoms between the oxygen atom and the NHBoc or NH2 group, which seems to correspond to the molecular formula of the commercial compound used. In the next figures (5 and 6), there are only 2 carbon atoms in the chain. This has to corrected."

What a fantastic catch! Thank you! We have now corrected the offending figures.

"It is not said anywhere if PODS absorb at 280 nm, and if so what is its molecular absorption coefficient at this wavelength. This is important to know for the precise determination of the concentration."

Another excellent point! We have now added the following sentence to Step 4.6, *"NOTE: The molar absorption coefficient for PODS at 298 nm is 12,400 $cm^{-1}M^{-1}$."*

"Lines 379 to 382. It is stated that the only difference between the radioimmunoconjugates prepared using the reported PODS method and the classical maleimide approach, was the bioconjugation handle of the chelators. This

Phone: 212.896.0443 *e-mail: brian.zeglis@hunter.cuny.edu*

statement is not completely true since the PODS bifunctional chelating agent contains also a PhNCS linker, which is not the case for the maleimide derivative."

This is a good point! We have amended the sentence to reflect this (underlining added for emphasis): *"Considering that the principal difference between the two radioimmunoconjugates was the bioconjugation handle of the chelator, the increased stability of the PODS-thiol linkage is almost certainly responsible for this improved in vivo performance."*

Reviewer #2:

Suggestions for improvement:

We thank the reviewer for their careful attention to our manuscript and their suggestions as to how to improve it!

"1.1 what volume flask?"

We have clarified. Step 1.1 now reads (underlining added for emphasis), *"In a 10 mL round-bottom flask, dissolve 100 mg (0.517 mmol, 1 equivalent) of 5-(4-aminophenyl)-1,3,4-oxadiazole-2-thiol in 3 mL of methanol."*

"1.2 diisopropylethylamine (anhydrous or other requirement)?"

We have clarified. Step 1.2 now reads (underlining added for emphasis), *"To this solution, add 360 µL of diisopropylethylamine (DIPEA; 2.07 mmol; 4 equivalents; anhydrous) and a small magnetic stir bar. Cover the flask with a rubber stopper and stir the solution for 10 minutes at room temperature."*

"1.3 how to add CH3I, using glass syringe? Are vessels sealed?"

We have clarified. Step 1.3 now reads (underlining added for emphasis), *"1.3 Using a 1 mL glass syringe, poke a hole through the rubber stopper and quickly add 32 µL (0.517 mmol, 1 equivalent) of iodomethane to this mixture. Allow the sealed mixture to react for 45 minutes at room temperature."*

"1.4 at r.t.?"

We have clarified. Step 1.4 now reads (underlining added for emphasis), *"Using a rotary evaporator, set the water bath to 40 °C and slowly reduce the pressure to remove the solvent to afford a white solid."*

"1.5 what volume NaHCO3? Specify multiple washes as necessary."

We have clarified. Step 1.5 now reads (underlining added for emphasis), *"Dissolve the solid in 3 mL of ethyl acetate and wash at least three times with a 5 mL solution of 0.1 M sodium carbonate using a separatory funnel."*

"1.6 DI water is acidic. Will pH 7 really be reached? Is this done using pH paper or pH meter?"

We have clarified. Step 1.6 now reads (underlining added for emphasis), *"Collect the organic phase in a separatory funnel and wash it with water until the pH of the aqueous phase reaches 6.8-7.0 (using pH paper)."*

"1.8 use rotavap?"

We have clarified. Step 1.9 (formerly Step 1.8) now reads (underlining added for emphasis), *"Evaporate the volatiles using a rotary evaporator, a process which should produce the desired product as white needles."*

"2.1 what volume flask?"

We have clarified. Step 2.1 now reads (underlining added for emphasis), *"In a 25 mL round bottom flask, dissolve*

Phone: 212.896.0443 e-mail: brian.zeglis@hunter.cuny.edu

387 mg (0.92 mmol, 1.0 equivalent) of NBoc-N'-succinyl-4,7,10-trioxa-1,13-tridecanediamine in 10 mL of dichloromethane."

"2.2 Is the vessel sealed?"

We have clarified. Step 1.9 (formerly Step 1.8) now reads (underlining added for emphasis), *"To this solution, add 480 µL (2.76 mmol, 3 equivalents) of DIPEA, 264 mg (1.38 mmol; 1.5 equivalents) of N-ethyl-N'-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI), and 200 mg (0.97 mmol, 1.1 equivalents) of **1**. Seal the vessel with a glass stopper and let the reaction stir for 5 days at room temperature."*

"2.5 add filtration step"

We have clarified. Step 2.5 now reads (underlining added for emphasis), *"Collect the organic phase and add magnesium sulfate to remove any traces of water. Filter the mixture using a medium glass frit or filter paper."*

"2.7 how slow?"

We have clarified. Step 2.7 now reads (underlining added for emphasis), *"Re-dissolve this solid in 10 mL of ethyl acetate and precipitate the product via the gradual (e.g. 2 mL at a time) addition of 30 mL of cyclohexane."*

"3.1 what volume flask?"

We have clarified. Step 2.7 now reads (underlining added for emphasis), *"In a 10 mL round-bottom flask, dissolve 30 mg (0.05 mmol; 1 equivalent) of **2** in 4 mL of dichloromethane."*

"3.2 sealed?"

We have clarified. Step 3.2 now reads (underlining added for emphasis), *"Slowly add in 49 mg (0.2 mmol; 4 equivalents) of 70% m-chloroperbenzoic acid to this mixture and cover the reaction vessel with a glass stopper. Stir the solution overnight at room temperature, ultimately yielding a yellow mixture."*

"3.4 and filter"

We have clarified. Step 3.4 now reads (underlining added for emphasis), *"Dry the organic phase with magnesium sulfate and filter the mixture using a medium glass frit or filter paper."*

"4.1 what volume flask?"

We have clarified. Step 4.1 now reads (underlining added for emphasis), *"In a 25 mL round bottom flask, dissolve 30 mg of **3** in 2.0 mL of dichloromethane."*

"4.2 sealed?"

We have clarified. Step 4.2 now reads (underlining added for emphasis), *"Add 400 µL of trifluoroacetic acid and seal the flask with a glass stopper."*

"4.4 at r.t.?"

We have clarified. Step 4.4 now reads (underlining added for emphasis), *"Using a rotary evaporator, remove the volatiles under reduced pressure at room temperature, leaving an oily residue."*

Phone: 212.896.0443 e-mail: brian.zeglis@hunter.cuny.edu

"5.1 What volume vessel?"

We have clarified. Step 5.1 now reads (underlining added for emphasis), *"In a 1.5 mL microcentrifuge tube, dissolve 10 mg of PODS in 300 µL of dimethyl sulfoxide (0.018 mmol; 1 equivalent) and add 26 µL of N,N-diisopropylethylamine (0.15 mmol; 8 equivalents)."*

"5.2 sealed?"

We have clarified. Step 5.2 now reads (underlining added for emphasis), *"Dissolve 15.2 mg of DOTA-Bn-NCS (0.02 mmol; 1.2 equivalents) in 100 µL of dimethylsulfoxide and combine this solution with the solution from Step 1. Seal the microcentrifuge tube."*

"5.4 column dimensions and flow rate?"

We have clarified. Step 5.4 now reads (underlining added for emphasis), *"Purify the product using reversed-phase C_{18} HPLC chromatography to remove any unreacted DOTA-Bn-NCS. NOTE: Retention times are obviously highly dependent on the HPLC equipment of each laboratory (pumps, columns, tubing, etc.), and appropriate controls should be run prior to purification. However, to present an example, if a gradient of 5:95 MeCN/H_2O (both with 0.1% TFA) to 70:30 MeCN/H_2O (both with 0.1% TFA) over 30 min, a semi-preparative 19 x 250 mm C_{18} column, and a flow rate of 6 mL/min are used, PODS, DOTA, and PODS-DOTA will have retention times of around 14.4, 18.8, and 19.6 min, respectively. All three compounds can be monitored at 254 nm."*

"6.1 Phosphate buffered saline or phosphate buffer solution? In what flask?"

We have clarified. Step 6.1 now reads (underlining added for emphasis), *"In a LoBind 1.5 mL microcentrifuge tube, dilute 61 µL of the trastuzumab stock solution (1 mg; 6.67 nmol, 1 equivalent) with 859 µL of phosphate buffered saline (pH 7.4)."*

"6.4 sealed or capped?"

We have clarified. Step 6.1 now reads (underlining added for emphasis), *"Seal the microcentrifuge tube and incubate the solution for 2 hours at room temperature."*

"6.5 how to monitor purification?"

Unfortunately, there is no way to monitor the purification of the immunoconjugate in real-time.

"Where is evidence for site-specific modification of the antibody?"

The root of the site-selective modification of the antibody lies in (a) PODS can only react with thiols and (b) there are only thiols in certain positions of human and murine IgGs. We have discussed this in the introduction, writing, *"IgG1 antibodies naturally contain 16 intra- and inter-chain disulfide bridges, linkages that can be selectively reduced to yield free thiols capable of undergoing Michael addition reactions with maleimides to form succinimidyl thioether bonds."* In light of this comment, we have come to the conclusion that "site-selective" is a better term than "site-specific" to describe our approach to bioconjugation. As a result, we have changed all references to site-specificity throughout the manuscript to site-selectivity.

"How does one characterize the degree of modification of the antibody?"

We have already stated this in the "Representative Results": *"MALDI-ToF analysis revealed a degree of labeling (DOL) of ~2 DOTA/mAb."*

Phone: 212.896.0443 *e-mail: brian.zeglis@hunter.cuny.edu*

Reviewer #3:

"The manuscript "Synthesis and Bioconjugation of Thiol Reactive Reagents for the Creation of Site-Selectively Modified Immunoconjugates" provides a method for the site-selective conjugation to native antibodies. The authors describe the construction of a reagent (PODS) and the subsequent generation of a chelating payload (PODS-DOTA). Finally the authors describe a facile method for the reduction and conjugation of PODS-DOTA to reduced cysteines in the antibody Trastuzumab. The in vivo stability of conjugates has been an ongoing challenge in the construction of antibody conjugates to reduce payload loss and improve specificity of signal. Indeed the manuscript also presents compelling in vivo examples showing reduced background of the PODS-DOTA armed antibodies. Overall the manuscript is well written and presented and provides information that is of interest to a wide range of areas."

We thank the reviewer for their kind words regarding our manuscript.

"Radiolabelling of antibodies has conventionally been done using conjugations to surface exposed lysines. The conjugation chemistry here is also stable. The manuscript may benefit from mentioning this in the discussion and highlighting the advantages of the presented technology over this conventional method."

This is a good point. We have already addressed this in brief, both in the "Introduction": ***"****Far and away the most common approach to the radiolabeling of antibodies is predicated on the indiscriminate attachment of radiolabeled prosthetic groups or radiometal chelators to amino acids — most often lysines — within the structure of the immunoglobulin (Figure 1A).*[2] *While this strategy is certainly effective, its random, non-site-specific nature can create problems. Specifically, traditional bioconjugation approaches produce poorly-defined and heterogeneous immunoconjugates composed of mixtures of thousands of different regioisomers, each with its own set of biological and pharmacological properties.*[3] *Furthermore, random bioconjugation can impede the immunoreactivity of antibodies if the cargo is appended to the immunoglobulin's antigen-binding domains."*

In addition, we tackle this topic briefly in the "Discussion": *"Taking a broader view, the benefits of site-selective bioconjugation have been illustrated repeatedly for both radioimmunoconjugates and antibody-drug conjugates.*[8,14,25-30] *In short, not only do site-selective bioconjugation strategies produce more well-defined and homogenous immunoconjugates than traditional methodologies, they also create imaging agents, radioimmunotherapeutics, and ADCs with improved in vivo performance."*

In response to this critique, we have added a few more sentences to the "Discussion" to drive home the point about the inadequacy of random bioconjugation: *"The non-site-selective bioconjugation of probes to lysines within antibodies is admittedly a straightforward and facile approach to the modification of antibodies. However, the presence of multiple lysines distributed throughout the structure of immunoglobulins means that it is impossible to exert control over the precise site or degree of bioconjugation. Predictably, then, this strategy often produces poorly-defined and highly heterogeneous immunoconjugates that can exhibit decreased immunoreactivity if ligations occur within the antigen-binding domains."*

Thank you for your time, work, and consideration. Please let us know if you require any more information.

Respectfully,

Brian M. Zeglis, Ph.D.

Phone: 212.896.0443 *e-mail: brian.zeglis@hunter.cuny.edu*