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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*

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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

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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

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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*

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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."*

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"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."*

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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,

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