



HAL
open science

Moving Beyond Isothiocyanates: A Look at the Stability of Conjugation Links Toward Radiolysis in ^{89}Zr -Labeled Immunoconjugates

Romane Vizier, Pierre Adumeau, Mathieu Moreau, Victor Goncalves, Franck Denat

► To cite this version:

Romane Vizier, Pierre Adumeau, Mathieu Moreau, Victor Goncalves, Franck Denat. Moving Beyond Isothiocyanates: A Look at the Stability of Conjugation Links Toward Radiolysis in ^{89}Zr -Labeled Immunoconjugates. *Bioconjugate Chemistry*, In press, <10.1021/acs.bioconjchem.4c00105>. <hal-04562036>

HAL Id: hal-04562036

<https://hal.science/hal-04562036v1>

Submitted on 30 Apr 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC-ND 4.0 - Attribution - Non-commercial use - No Derivative Works - International License

Moving beyond isothiocyanates: A look at the stability of conjugation links towards radiolysis in ⁸⁹Zr-labeled immunoconjugates

Romane Vizier, Pierre Adumeau*, Mathieu Moreau, Victor Goncalves, Franck Denat*

Institut de Chimie Moléculaire de l'Université de Bourgogne, UMR 6302, Université de Bourgogne, CNRS, Dijon, France

Corresponding authors :

Prof. Franck Denat, 9 Avenue Alain Savary, 21000 Dijon, France, email: franck.denat@u-bourgogne.fr, phone: +33 380 396 115.

Dr. Pierre Adumeau, Current address : 20 Rue Jean Mazen, 21000 Dijon, France, email : padumeau@oncodesign-services.com.

ABSTRACT

Zirconium-89 is the most widely used radioisotope for immunoPET because its physical half-life (78.2 hours) suits the one of antibodies. Desferrioxamine B (DFO) is the standard chelator for the complexation of zirconium(IV) and its bifunctional version, containing a phenylisothiocyanate function, is the most commonly used for the conjugation of DFO to proteins. However, preliminary results have shown that the thiourea link obtained from the conjugation of isothiocyanate and lysines is sensitive to the ionizing radiations generated by the radioisotope, leading to the rupture of the link and the release of the chelator/radiometal complex. This radiolysis phenomenon could produce non-specific signal and prevent the detection of bone metastasis, as free zirconium accumulates into the bones. The aim of this work was to study the stability of a selection of conjugation linkers in ⁸⁹Zr-labeled immunoconjugates. We have synthesized several DFO-based bifunctional chelators appended with an isothiocyanate moiety, a bicyclononyne or a squaramate ester. Two antibodies (trastuzumab and rituximab) were conjugated and radiolabeled with zirconium-89. The effect of increasing activities of zirconium-89 on the integrity of the bioconjugate bearing thiourea links was evaluated, as well as the impact of the presence of a radioprotectant. The stability of the radiolabeled antibodies was studied over 7 days in PBS and in human plasma. Radioconjugates' integrity was evaluated using iTLC and size-exclusion chromatography. This

study shows that the nature of the linker between the chelator and biomolecule can have a strong impact on the stability of the ^{89}Zr -labeled conjugates, as well as on the aggregation of the conjugates.

KEYWORDS

Radiolysis; ImmunoPET; zirconium-89; SEC-HPLC; DFO; Conjugation link

INTRODUCTION

Over the past two decades, ^{89}Zr -ImmunoPET has shown great potential as a diagnostic tool for cancer imaging.^{1,2} The physical half-life of ^{89}Zr (78.4 h) matches the biological half-life of IgG1 monoclonal antibodies (mAbs). ^{89}Zr -labeled antibodies can for example be used in preclinical studies to assess the biodistribution of novel mAbs. More importantly, ^{89}Zr -ImmunoPET could help in clinical practice, to help understanding heterogeneity in mAb uptake, predict tumor response and serve as a diagnostic companion to select patients able to respond to immunotherapies, ADC therapies, targeted radiotherapies, and to monitor these treatments.^{3,4}

Besides the affinity of these radiolabeled antibodies for the target, an obvious requirement to ensure that PET images accurately reflect the bioconjugates biodistribution is that radioisotope and protein remain linked together. In this respect, a crucial parameter is the stability of the ^{89}Zr complex, to avoid the release of ^{89}Zr *in vivo*, either by transmetallation or transchelation phenomena. The current standard chelator for ^{89}Zr radiolabeling is desferrioxamine (DFO). However, due to suboptimal coordination properties, the ^{89}Zr complex formed with this natural siderophore is not highly stable, and this results in some ^{89}Zr release *in vivo*, which was evidenced by significant uptake of radioactivity in bones, at least in mice, in several preclinical studies.^{5,6} This drawback prompted several research groups, including ours, to design and evaluate new chelators, capable of binding more strongly the radiometal cation.⁷⁻⁹

In addition to the inertness of the metal complex, another key point is the chemical stability of the whole conjugate. In particular, the metal complex must be strongly and stably bonded to the biovector, in order to prevent a release of the radioactive complex from the targeting vehicle, as this radioactive complex will distribute differently *in vivo* and provide erroneous biodistribution results. It is well established that emissions from the radionuclide can produce reactive oxygen species (ROS) such as hydroxyl or superoxide anion radicals that can break chemical bonds.¹⁰⁻¹⁴ This radiolysis phenomenon is known as one of the main factors for the degradation of radiopharmaceuticals, and can be partially circumvented by using radioprotectants such as sodium ascorbate, gentisic acid, *N*-acetyl-L-cysteine, ethanol or methionine, either separately or in combination.¹³ However, the development of these formulations remains empirical and varies according to numerous parameters such as the nature of the vector, the radionuclide, the chelator and the linker between the vector and the chelator, but also – if not mostly – the habits of the research groups. Although the formulation of radiopharmaceuticals used in clinical practice is well defined, no single solution is valid for all of them.¹⁵ More surprisingly, there are only very few studies reporting a potential relation between the extend of radiolysis and the nature of the bioconjugation link.^{11,12,16,17} In order to fill part of this gap, we aimed in this work at comparing the stability of different conjugation links in ^{89}Zr -labeled immunoconjugates. Although not optimal, Desferrioxamine (DFO) was chosen as ^{89}Zr chelator because it is the standard so far for the synthesis of ^{89}Zr -labeled immunoconjugates. Its bifunctional version containing a phenylisothiocyanate function (DFO-*p*Phe-NCS) is the most commonly used for the conjugation to proteins.¹² The reaction of NCS

group with the primary amine of lysines results in the formation of a thiourea bond. Many other bifunctional versions of DFO have been reported,⁷ among which we chose DFO-squaramate ethyl ester¹⁸ and DFO-bicyclononyne (BCN),¹⁹ resulting after conjugation in a squaramide link and a triazole ring, respectively.

RESULTS AND DISCUSSION

Synthesis of the immunoconjugates

Starting from a commercially available DFO precursor, DFO mesylate (DFO.Mes), we prepared three different bifunctional versions: DFO-*p*Phe-NCS, DFO-squaramate ethyl ester and DFO-BCN, by reacting DFO.Mes with *p*-phenylene diisothiocyanate (PDITC), diethylsquarate and bicyclononyne *p*-nitrophenyl carbonate, respectively (Figure 1).

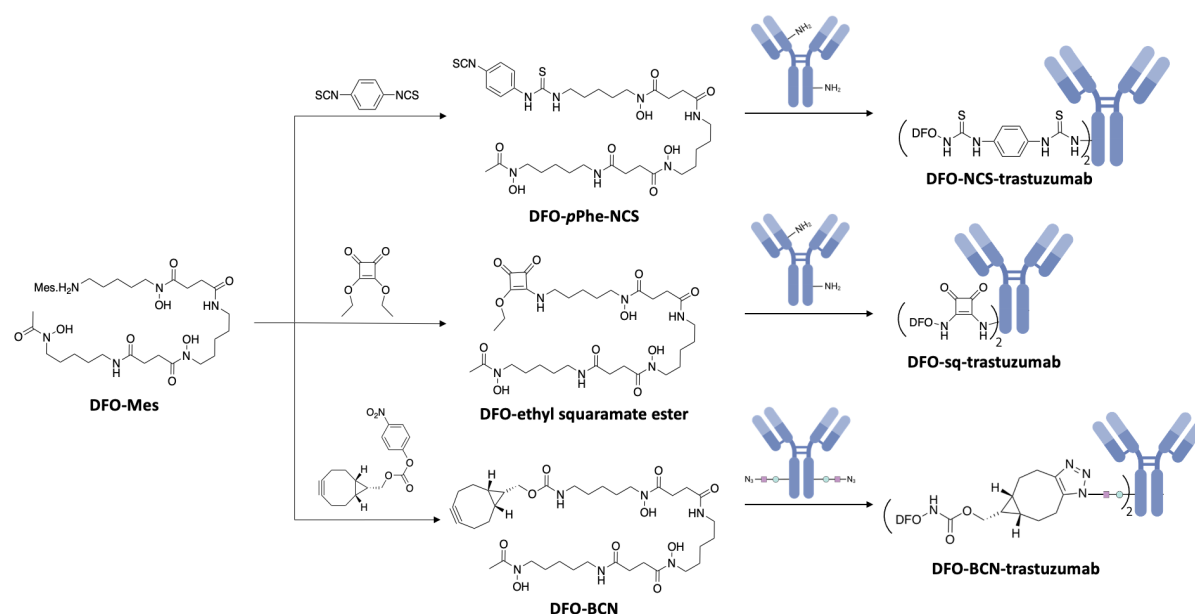


Figure 1. Synthesis of the three bioconjugates

The three bifunctional chelating agents were then conjugated to trastuzumab (Figure 1). Random conjugation of DFO-*p*Phe-NCS and DFO-squaramate ethyl ester to lysine residues was performed by incubating the DFO derivatives with the antibody 1 hour at 37°C. The conditions were optimized to obtain an average degree of labeling (DOL, or chelator/antibody ratio) close to 2. DFO-BCN was conjugated site-specifically, via the reaction of the bifunctional chelating agent with the antibody previously modified with two azide groups using glycan engineering.^{19,20} The strain-promoted azide-alkyne cycloaddition (SPAAC) results in the formation of a triazole ring.

Radiolabeling

The three immunoconjugates DFO-NCS-trastuzumab, DFO-sq-trastuzumab and DFO-BCN-trastuzumab, were radiolabeled with ^{89}Zr to reach a specific activity (SA) at 60 MBq/mg. For DFO-NCS-trastuzumab, radiolabelings with ^{89}Zr at 15, 30, and 120 MBq/mg were also performed. Radiochemical yields and radiochemical purity were determined by iTLC (> 98.5 % for all compounds).

Study of the stability of the radiolabeled immunoconjugates

First we studied the stability of $[^{89}\text{Zr}]\text{Zr}$ -DFO-NCS-trastuzumab by SEC-HPLC. Whereas the iTLC method - routinely used for the evaluation of the radiochemical purity and stability of radioconjugates - is mostly informative on free $[^{89}\text{Zr}]\text{Zr}^{4+}$ and, to some extent, on small $[^{89}\text{Zr}]\text{Zr}$ -labeled compounds, the SEC-HPLC technique allows the separation of the ^{89}Zr -labeled antibody from smaller ^{89}Zr -labeled compounds that could result from the degradation of the conjugate, as well as higher molecular weight (HMW) species caused by the aggregation of the radiolabeled protein on itself or with plasma proteins (Figure S1).

At low specific activities (up to SA = 30 MBq/mg), the radiolabeled conjugate remained stable after one week in PBS (Figure 2). However, when increasing the activity, rapid degradation was observed with only ca. 50% and 25% of the radiolabeled antibody remaining intact after 3 days at SA = 60 and 120 MBq/mg respectively. Interestingly, this phenomenon is not evidenced by iTLC analysis with the standard elution conditions used for radioconjugates (elution with EDTA pH 5.5), hinting that the small ^{89}Zr -labeled species observed by SEC-HPLC are not due to the release of $[^{89}\text{Zr}]\text{Zr}^{4+}$ from the chelator (Figure S2). These results suggest that the degradation is due to radiolysis phenomenon.

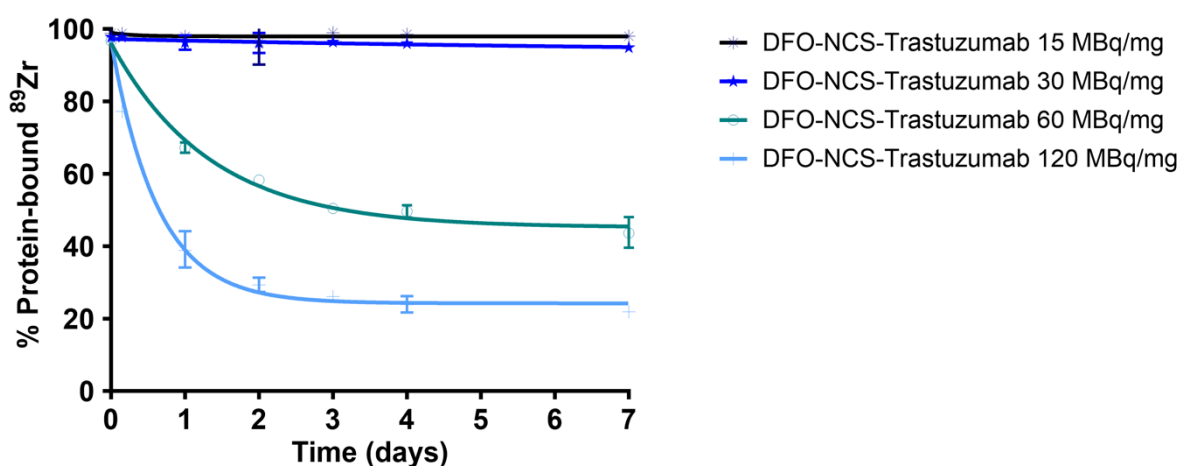


Figure 2. Stability of $[^{89}\text{Zr}]\text{Zr}$ -DFO-NCS-trastuzumab in PBS by SEC-HPLC with different specific activities ($n=3$)

In a second set of experiments, we compared the stability of the three radiolabeled conjugates (SA = 60 MBq/mg) over a period of one week in PBS (Figure 3a). The results showed that $[^{89}\text{Zr}]\text{Zr}$ -DFO-sq-trastuzumab and $[^{89}\text{Zr}]\text{Zr}$ -DFO-BCN-trastuzumab were much more stable when compared to $[^{89}\text{Zr}]\text{Zr}$ -

DFO-NCS-trastuzumab, with only a small amount (ca. 20%) of ^{89}Zr -DFO complex released after 7 days. This release was significantly reduced for all conjugates when the PBS was supplemented with the radical scavenger *N*-acetyl-L-cysteine (NAC), an antioxidant demonstrating excellent radioprotectant effect on [^{89}Zr]Zr-labeled antibodies,¹⁰ confirming that the observed degradation is indeed linked to radiolysis (Figure 3a). The similar behavior of [^{89}Zr]Zr-DFO-sq-trastuzumab and [^{89}Zr]Zr-DFO-BCN-trastuzumab suggests that the stability is not related to whether bioconjugation is site-specific or not, and that the increased stability is more likely due to the higher inertness of the chemical link between the ^{89}Zr complex and the protein compared to [^{89}Zr]Zr-DFO-NCS-trastuzumab.

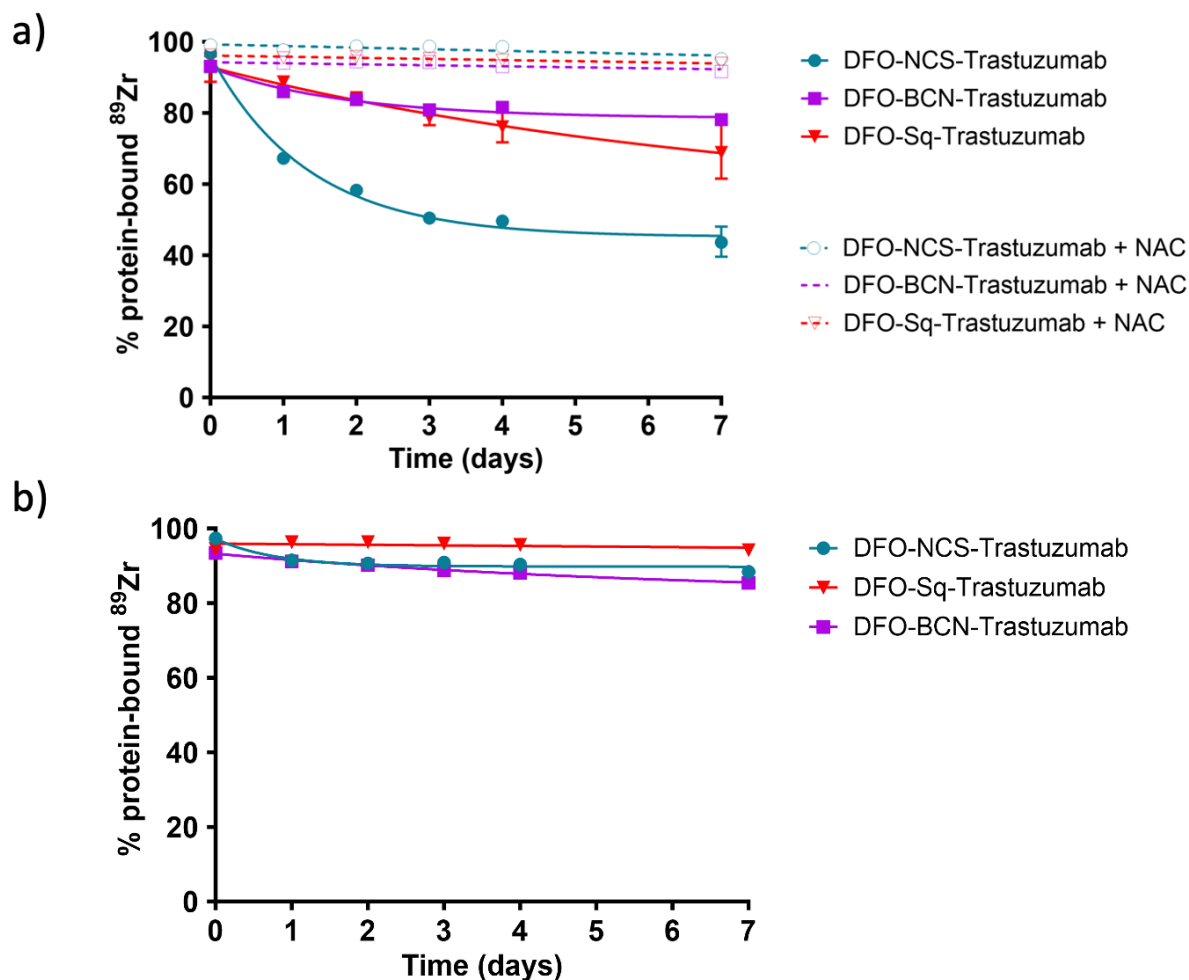


Figure 3. (a) Stability of the three radioconjugates in PBS, or in PBS supplemented with *N*-acetyl-L-cysteine (NAC, 0.5 g.L⁻¹), by SEC-HPLC, SA=60 MBq/mg, n=3 (b) Stability of the three radioconjugates in plasma by SEC-HPLC, SA=60 MBq/mg, n=3

Stability studies were also carried out with another model antibody, rituximab, a chimeric monoclonal antibody targeted against CD20, which was randomly conjugated to DFO-*p*Phe-NCS and DFO-squaramate ethyl ester. Mirroring the results obtained with trastuzumab, we observed superior stability of the squaramide derivative versus the phenyldithiourea derivative in PBS with respect to radiolysis (Figure S3).

In order to probe for detrimental repercussion *in vivo*, these stability studies were then repeated in plasma. Interestingly, we observed a very limited release of low molecular weight radioactive species, regardless of the nature of the linker, almost all ^{89}Zr remaining protein bound (Figure 3b). The plasma probably contains certain species, such as vitamins, enzymes and glutathione, which behave as natural radicals scavengers and prevent radiolysis effects that are responsible of the cleavage of the link.^{21,22} However, we noticed the progressive formation of high molecular weight species (reaching ca 20% after 7 days) for trastuzumab-NCS-DFO, suggesting aggregation of this product in plasma, possibly with endogenous plasma proteins (Figure 4). No such phenomenon was observed for the other two conjugates. Thus, in addition to their better resistance to radiolysis, the use of triazole and squaramide linkers is beneficial compared with the phenyldithiourea group.

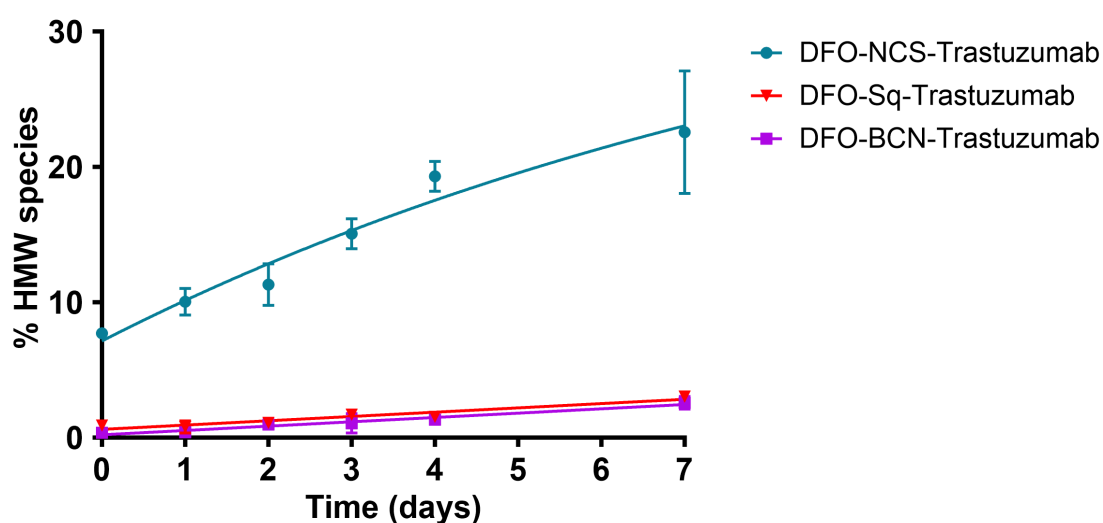


Figure 4. Formation of high molecular weight species (HMW) in plasma for the three radioconjugates, by SEC-HPLC, SA=60 MBq, n=3

Liu *et al.* hypothesized a release of ^{90}Y -DOTA complex resulting from the radiolysis of a DNA analog conjugated to *p*-SCN-Bz-DOTA.¹⁷ More recently, Aluicio-Sardy *et al.* observed the radiolysis of a PSMA targeting agent (DUPA) radiolabeled with a $^{132/135}\text{La}$ -macropa complex, while the same ligand conjugated to a DO2Apic chelator was stable.¹⁶ The chelating agents macropa and DO2Apic were attached to the ligand through a thiourea bond and an amide bond respectively. The authors suggested that the decomposition products arise from the radiolysis of the aromatic thiourea linkage, although this was not further investigated. In a head-to-head comparison of four different ^{89}Zr bifunctional chelating agents conjugated to trastuzumab (using two different chelators, DFO and DFO*, and two different conjugation groups, NCS and squarate), Chomet *et al.* reported a relative instability of [^{89}Zr]Zr-DFO-NCS-trastuzumab, with a radiochemical purity dropping to ca. 70% after incubating 24 h in pH 7 buffer.²³ This can be partially solved by using the superior chelating agent DFO*, but the conjugates are

still less stable than the analogs conjugated through the squaramate. Last but not least, in the first article reporting DFO-*p*Phe-NCS, the authors warned on the need to store the conjugate under specific conditions, to avoid its radiolysis.¹² These results from the literature support our findings that the thiourea bond is not as stable as amide bonds or other linkages such as the triazole ring towards radiolysis. In fact, this is not so surprising since it has been shown that thiourea derivatives are prone to radiolysis²⁴ and early articles reported the use of thiourea as a radioprotectant due to its role as a scavenger of ROS.²⁵

In order to try to understand why such instability of the thiourea bond towards radiolysis in radioconjugates has not been reported more extensively so far, we decided to investigate the stability of the three radioconjugates by iTLC using EDTA 0.1 M solution as eluent. Unlike what was observed by SEC-HPLC, all tested radioconjugates seem stable after a one-week incubation in PBS (Figure S2). This is due to the inability of this iTLC method to separate the ⁸⁹Zr-labeled conjugate from the cleaved ⁸⁹Zr complex, both remaining at the spotting site.

As iTLC has long been the standard method for assessing the radiochemical purity of radiopharmaceuticals, this could explain why the cleavage of the bioconjugation bond was not systematically observed in previous work, and why the NCS group is still so commonly used nowadays for the synthesis of radioconjugates.²⁶ Besides the fact that alternatives to the thiourea bond should be preferred for the design of radiopharmaceuticals, these results show that SEC-HPLC is a more reliable method than iTLC for the evaluation of radiochemical purity and stability of radiolabeled compounds.

CONCLUSION

Although the effect of radiolysis on the stability of radiolabeled immunoconjugates can be effectively mitigated by the use of radioprotectants, the optimization of anti-radiolytic formulation is still empirical. Therefore, it is of major importance to synthesize conjugates as stable as possible, and particular attention must be paid to the antibody labeling, not only to the choice of the chelator but also to the selection of the bioconjugation method. In this work, we have demonstrated that the isothiocyanate group, which is currently the standard method for the random conjugation of various cargos to lysine residues of antibodies, can be advantageously replaced with a squaramate ester for the attachment of radiometal chelators to lysines. We have also shown that the triazole ring resulting from the SPAAC reaction between an azide and a constraint alkyne is resistant towards radiolysis, which is very positive since click chemistry has proven to be a powerful tool for site-specific labeling of antibodies to construct more homogenous bioconjugates. It has also been proven that this relative instability of the thiourea bond towards radiolysis was apparently not antibody-dependent. Finally, we have also noticed that the conjugate prepared using DFO-*p*Phe-NCS was more prone to aggregation phenomena when compared to the other two conjugates. We believe that these findings should help in the future to design optimized ⁸⁹Zr labeled mAbs for immunoPET.

MATERIAL AND METHODS

A detailed description of experimental procedures – including synthetic protocols, bioconjugation techniques, radiolabeling methods, and analytical techniques can be found in the Supplementary Information.

FUNDING SOURCES

The Ministère de l'Enseignement Supérieur et de la Recherche, the Centre National de la Recherche Scientifique (CNRS), the Conseil Régional de Bourgogne (PhD ICE grant), are gratefully acknowledged. This work is part of the project "Pharmaco-imagerie et agents théranostiques" supported by the Université de Bourgogne and the Conseil Régional de Bourgogne Franche-Comté through the "Plan d'Action Régional pour l'Innovation" (program PARI) and by the European Union through the PO FEDER-FSE 2014/2020 Bourgogne program. Support was also provided by the French Government through the French National Research Agency (ANR) under the program "Investissements d'Avenir" (ANR-10-EQPX-05-01/ IMAPPI Equipex).

ACKNOWLEDGMENTS

The authors thank the "Plateforme d'Analyse Chimique et de Synthèse Moléculaire de l'Université de Bourgogne" (PACSMUB, <http://www.wpcm.fr>) for access to analytical instrumentation, and Marie-José Penouilh (University of Burgundy, PACSMUB) and Dr. Quentin Bonnin (University of Burgundy, PACSMUB) for the high-resolution mass spectrometry. The Table of Contents graphic was created with BioRender.com.

SUPPORTING INFORMATION DESCRIPTION

Materials and methods, experimental details on chemical synthesis and immunoconjugation, radiolabeling, *in vitro* stability. Additional figures on SEC-HPLC analyses and stability of radioimmunoconjugates.

ABBREVIATIONS

BCN: (1R,8S,9S)-bicyclo[6.1.0]non-4-yne; DFO: desferrioxamine B; DOL: degree of labeling; EDTA: ethylenediaminetetraacetic acid; iTLC: instant thin layer chromatography; NAC: *N*-acetyl-L-cysteine; PBS: phosphate buffer saline; PET: positron emission tomography; SA: specific activity; SEC-HPLC : size exclusion high performance liquid chromatography; SPAAC: strain-promoted alkyne-azide cycloaddition.

REFERENCES

1. De Feo MS, Pontico M, Frantellizzi V, Corica F, De Cristofaro F, De Vincentis G. ⁸⁹Zr-PET imaging in humans: a systematic review. *Clin Transl Imaging*. 2022;10:23-36.
2. van Dongen GAMS, Beaino W, Windhorst AD, Zwezerijnen GJC, Oprea-Lager DE, Hendrikse NH, van Kuijk C, Boellaard R, Huisman MC, Vugts DJ. The Role of ⁸⁹Zr-Immuno-PET in Navigating and Derisking the Development of Biopharmaceuticals. *J Nucl Med*. 2021;62:438-445.
3. Yoon J-K, Park B-N, Ryu E-K, An Y-S, Lee S-J. Current Perspectives on ⁸⁹Zr-PET Imaging. *International Journal of Molecular Sciences*. 2020;21:4309.
4. McKnight BN, Viola-Villegas NT. ⁸⁹Zr-ImmunoPET Companion Diagnostics and their Impact in Clinical Drug Development. *J Labelled Comp Radiopharm*. 2018;61:727-738.
5. Perk LR, Visser GWM, Vosjan MJWD, Stigter-van Walsum M, Tjink BM, Leemans CR, van Dongen GAMS. ⁸⁹Zr as a PET Surrogate Radioisotope for Scouting Biodistribution of the Therapeutic Radiometals ⁹⁰Y and ¹⁷⁷Lu in Tumor-Bearing Nude Mice After Coupling to the Internalizing Antibody Cetuximab. *J Nucl Med*. 2005;46:1898-1906.
6. Dijkers ECF, Kosterink JGW, Rademaker AP, Perk LR, van Dongen GAMS, Bart J, de Jong JR, de Vries EGE, Lub-de-Hooge MN. Development and Characterization of Clinical-Grade ⁸⁹Zr-Trastuzumab for HER2/neu ImmunoPET Imaging. *Journal of Nuclear Medicine*. 2009;50:974-981.
7. Heskamp S, Raavé R, Boerman O, Rijpkema M, Goncalves V, Denat F. ⁸⁹Zr-Immuno-Positron Emission Tomography in Oncology: State-of-the-Art ⁸⁹Zr Radiochemistry. *Bioconjugate Chem*. 2017;28:2211-2223.
8. Bhatt NB, Pandya DN, Wadas TJ. Recent Advances in Zirconium-89 Chelator Development. *Molecules*. 2018;23:638.
9. Raavé R, Sandker G, Adumeau P, Borch Jacobsen C, Mangin F, Meyer M, Moreau M, Bernhard C, Da Costa L, Dubois A, et al. Direct comparison of the in vitro and in vivo stability of DFO, DFO* and DFOcyclo* for ⁸⁹Zr-immunoPET. *Eur J Nucl Med Mol Imaging*. 2019;46:1966-1977.
10. Bhatt NB, Pandya DN, Rideout-Danner S, Gage HD, Marini FC, Wadas TJ. A comprehensively revised strategy that improves the specific activity and long-term stability of clinically relevant ⁸⁹Zr-immuno-PET agents. *Dalton Trans*. 2018;47:13214-13221.
11. Sharma SK, Glaser JM, Edwards KJ, Khozeimeh S, Salih AK, Lewis JS, Price EW. A Systematic Evaluation of Antibody Modification and ⁸⁹Zr-Radiolabeling for Optimized

Immuno-PET. *Bioconjugate Chem.* 2021;32:1177-1191.

12. Perk LR, Vosjan MJWD, Visser GWM, Budde M, Jurek P, Kiefer GE, van Dongen GAMS. p-Isothiocyanatobenzyl-desferrioxamine: a new bifunctional chelate for facile radiolabeling of monoclonal antibodies with zirconium-89 for immuno-PET imaging. *Eur J Nucl Med Mol Imaging.* 2010;37:250-259.
13. de Zanger RMS, Chan HS, Breeman WAP, de Blois E. Maintaining radiochemical purity of [¹⁷⁷Lu]Lu-DOTA-PSMA-617 for PRRT by reducing radiolysis. *J Radioanal Nucl Chem.* 2019;321:285-291.
14. Liu S, Edwards DS. Stabilization of ⁹⁰Y-Labeled DOTA-Biomolecule Conjugates Using Gentisic Acid and Ascorbic Acid. *Bioconjugate Chem.* 2001;12:554-558.
15. Larenkov A, Mitrofanov I, Pavlenko E, Rakhimov M. Radiolysis-Associated Decrease in Radiochemical Purity of ¹⁷⁷Lu-Radiopharmaceuticals and Comparison of the Effectiveness of Selected Quenchers against This Process. *Molecules.* 2023;28:1884.
16. Aluicio-Sarduy E, Thiele NA, Martin KE, Vaughn BA, Devaraj J, Olson AP, Barnhart TE, Wilson JJ, Boros E, Engle JW. Establishing Radiolanthanum Chemistry for Targeted Nuclear Medicine Applications. *Chemistry – A European Journal.* 2020;26:1238-1242.
17. Liu C, Liu G, Liu N, Zhang Y, He J, Rusckowski M, Hnatowich DJ. Radiolabeling morpholinos with ⁹⁰Y, ¹¹¹In, ¹⁸⁸Re and ^{99m}Tc. *Nuclear Medicine and Biology.* 2003;30:207-214.
18. Rudd SE, Roselt P, Cullinane C, Hicks RJ, Donnelly PS. A desferrioxamine B squaramide ester for the incorporation of zirconium-89 into antibodies. *Chem Commun.* 2016;52:11889-11892.
19. Adumeau P, Raavé R, Boswinkel M, Heskamp S, Wessels HJCT, van Gool AJ, Moreau M, Bernhard C, Da Costa L, Goncalves V, Denat F. Site-Specific, Platform-Based Conjugation Strategy for the Synthesis of Dual-Labeled Immunoconjugates for Bimodal PET/NIRF Imaging of HER2-Positive Tumors. *Bioconjugate Chem.* 2022;33:530-540.
20. Vivier D, Fung K, Rodriguez C, Adumeau P, Ulaner GA, Lewis JS, Sharma SK, Zeglis BM. The Influence of Glycans-Specific Bioconjugation on the FcγRI Binding and *In vivo* Performance of ⁸⁹Zr-DFO-Pertuzumab. *Theranostics.* 2020;10:1746-1757.
21. Balasaheb Nimse S, Pal D. Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Advances.* 2015;5:27986-28006.
22. Roche M, Rondeau P, Singh NR, Tarnus E, Bourdon E. The antioxidant properties of serum albumin. *FEBS Lett.* 2008;582:1783-1787.

23. Chomet M, Schreurs M, Bolijn MJ, Verlaan M, Beaino W, Brown K, Poot AJ, Windhorst AD, Gill H, Marik J, et al. Head-to-head comparison of DFO* and DFO chelators: selection of the best candidate for clinical ⁸⁹Zr-immuno-PET. *Eur J Nucl Med Mol Imaging*. 2021;48:694-707.
24. Dey GR, Naik DB, Kishore K, Moorthy PN. Nature of the transient species formed in the pulse radiolysis of some thiourea derivatives. *J Chem Soc, Perkin Trans 2*. 1994:1625-1629.
25. Charlesby A, Kopp PM, Read JF, Jackson W. Radiolysis of dilute aqueous thiourea solutions. *Proceedings of the Royal Society of London Series A Mathematical and Physical Sciences*. 1997;292:122-133.
26. Hooijman EL, Ntihakose CM, Reuvers TGA, Nonnekens J, Aalbersberg EA, van de Merbel JRJP, Huijmans JE, Koolen SLW, Hendrikx JJMA, de Blois E. Radiolabeling and quality control of therapeutic radiopharmaceuticals: optimization, clinical implementation and comparison of radio-TLC/HPLC analysis, demonstrated by [¹⁷⁷Lu]Lu-PSMA. *EJNMMI Radiopharmacy and Chemistry*. 2022;7:29.

