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COMMUNICATION

Scaling-down Antibody Radiolabeling Reactions with Zirconium-89

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The most widely cited procedures for radiolabeling antibodies with zirconium-89 for immuno-PET require multi-milligram amounts of antibody which can be cost-prohibitive, particularly during the research and development process. We therefore sought to develop a reliable ^{89}Zr-radiolabeling procedure that provides high radiochemical yields at the microgram scale.

INTRODUCTION

Interest in the positron emitting radiometal zirconium-89 has increased substantially over the last decade which is attributable to its advantageous properties for immuno-PET applications.[1-3] Its radioactive half-life ($t_{1/2}$: 78.42 h) is compatible with the slow clearance of antibodies from the circulation and allows PET images to be acquired when tumour-to-blood contrast ratios have reached satisfactorily high levels. The average positron emission energy of Zr-89 (395.5 keV) is low in comparison to other radiometals such as gallium-68 (836.02 keV) and copper-64 (653.03 keV) which contributes towards the acquisition of high resolution PET images.[4] Zr-89 also benefits from a residualizing effect which assists in the accumulation of radioactivity in tumorous tissue.

Over recent years, the process of attaching this radiometal to an antibody vector has undergone considerable refinement, however the general principle of first modifying the antibody with a chelating agent capable of forming a stable radiometal complex with zirconium-89 has remained unchanged. The chelating agent used almost exclusively for zirconium is the bacterial siderophore desferrioxamine (DFO; Fig. 1), although a few alternative chelators have recently shown some promise in this area.[5-8] One of the earliest approaches for modifying antibodies with DFO which became widely implemented involved forming an amide bond with a TFP-ester of an iron(III)-DFO complex (Fig. 1a).[9] In this procedure, the iron(III) is removed by competition with ethylenediaminetetraacetic acid (EDTA) prior to radiolabeling with Zr-89. More recently, a simpler method involving the bifunctional chelator *p*-SCN-Bn-desferrioxamine (*p*-SCN-Bn-DFO) has been developed in which the chelator becomes covalently tethered to the antibody *via* the formation of a stable thiourea group (Fig 1b).[10-12] Both of these methods have an unfavourable drawback in common which is the inability to control the precise location at which the DFO is attached to the antibody. While efforts are ongoing to develop superior site-selective modification of antibodies,[13] the random approach involving *p*-SCN-Bn-DFO remains at present the most popular method.

The three most widely cited methods in the literature for both DFO attachment and ^{89}Zr-radiolabeling of antibodies are highly comparable to each other and are summarised in Table 1. These methods all either explicitly recommend or at least describe the use of multi-milligram quantities of antibody. As the cost of many commercially available antibodies can frequently exceed several thousand US dollars for a single milligram, this represents a considerable financial hurdle particularly during early stages of research and development. To help reduce the cost for the financially prudent researcher, we sought to investigate how scaling down the quantity of antibody in these reactions might affect performance in terms of radiolabeling yield.

MATERIALS AND METHODS

General methods

All reagents and solvents were purchased from Sigma-Aldrich unless otherwise stated and were used without further purification. The chelating agent *p*-SCN-Bn-DFO was purchased from Macrocyclics Inc. (Dallas, TX). Water was deionised using a Barnstead NANOpure purification system (Thermo Scientific) and had a resistance of >18.2 MΩ cm^{-1} at 25°C. Protein

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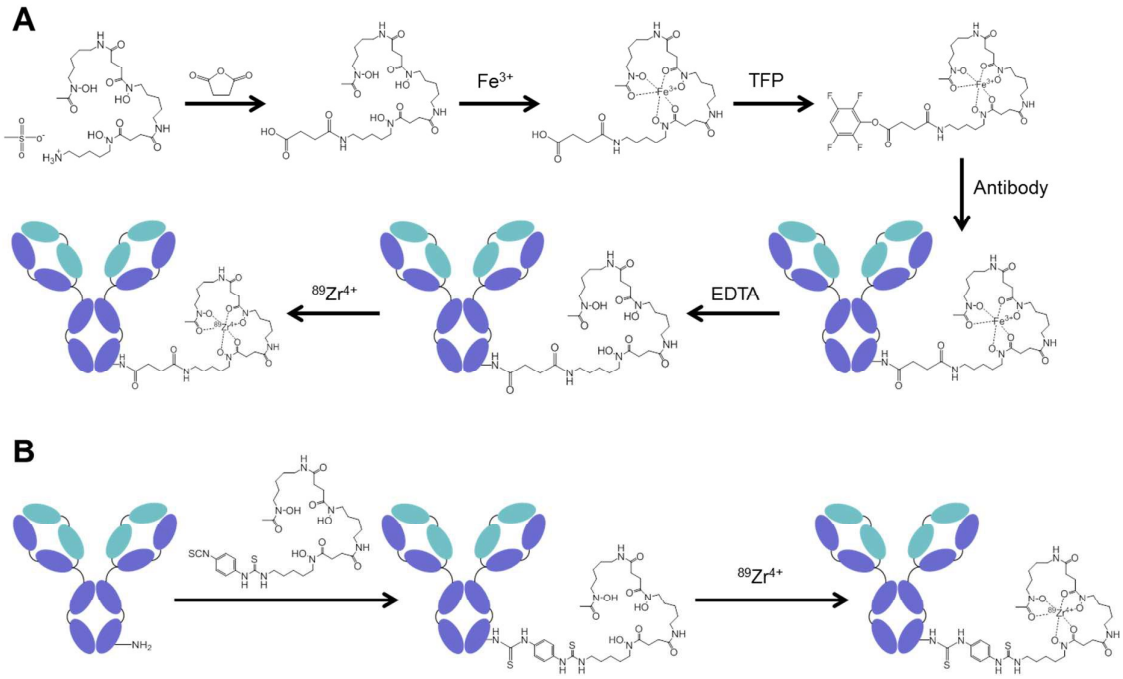

Fig. 1 Schemes of the two most common procedures for the production of ^{89}Zr radiolabeled antibodies for immuno-PET applications. **A**: The method reported by Verel *et al.* in 2003. **B**: The method described by Vosjan *et al.* in 2010 from which the work in this manuscript is derived.

concentration measurements were made on a ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). Instant thin-layer chromatography (iTLC) was performed on glass microfiber chromatography paper (Agilent Technologies) and strips were analysed with either a Bioscan AR-2000 radio-TLC scanner (Eckert & Ziegler) or a Cyclone Plus Phosphor Imager (PerkinElmer). pH measurements were determined using pH indicator paper (Merck Millipore) or a pH Spear electrode (Eutech Instruments). Radioactivity measurements were determined using a CRC®-25R dose calibrator (Capintec, Inc.). Radio-HPLC was carried out on an Agilent 1200 series machine equipped with a single wavelength UV detector set to 254 nm, a Lablogic Gamma-Ram 4 radiodetector and a Superdex 200 10/300 GL size exclusion column (GE Healthcare LifeSciences, cat. no. 17-5175-01). Samples were eluted over 60 minutes with PBS buffer pH 6.6. MALDI-TOF mass spectrometry (MS) data were obtained on a Waters MALDI Micro MX system in reflectron positive ion mode and samples were run with sinapinic acid.

Antibody modification with p-SCN-Bn-DFO

To a solution of tocilizumab (RoActemra®) or trastuzumab (Herceptin®) (500 µg) in 0.1 M $NaHCO_3$ (pH 8.9, 125 µL) was added 10 molar equivalents of p-SCN-Bn-DFO (6.64 mM) in anhydrous dimethyl sulfoxide. The volume of the p-SCN-Bn-DFO solution transferred to the antibody solution was kept below 5% (*v*/*v*). The reaction mixture was incubated at 37°C for 60 minutes with gentle shaking (450 rpm) and the excess p-SCN-Bn-DFO was removed by Sephadex-G50 size exclusion chromatography, eluting with 100 µL fractions of phosphate

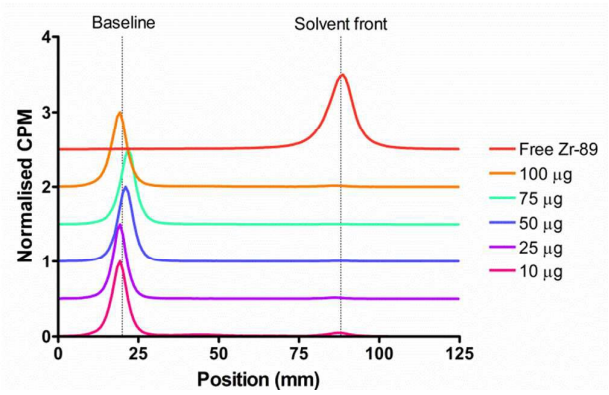

Fig. 2 Representative radio-TLCs of crude reaction mixtures after 1 h at room temperature. ^{89}Zr-labeled tocilizumab: R_f = 0; $^{89}Zr^{4+}$ ions and [^{89}Zr]-EDTA: R_f = 1

buffered saline (PBS, pH 7.4). After combining the fractions which corresponded to the DFO-modified antibody, the sample was washed twice with PBS (500 µL) and concentrated using an Amicon Ultra-0.5 mL centrifugal filter (30 kDa MWT cut-off, Merck Millipore). The average number of DFO species conjugated to each tocilizumab molecule was determined by both MALDI-TOF MS and isotopic dilution experiments following a method described by Holland *et al.*.[14]

Zirconium-89 radiolabeling procedure

Zirconium-89 in 1 M oxalic acid (sourced from VU Amsterdam, or PETIC Cardiff) was adjusted to pH 7-8 by the addition of 1 M sodium carbonate. The resulting solution was added to a 2 mg/mL solution of DFO-tocilizumab to achieve a ratio of 0.1 MBq to 1 microgram of antibody. The reaction mixtures were

Table 1 A selection of reaction parameters for both DFO-modification and ^{89}Zr-radiolabeling of antibodies

Procedure for modifying antibody with DFO								
Method	**Brief description**	**Initial amount of antibody (mg)**	**Molar excess of DFO species**	**Buffer**	**pH**	**Temperature**	**Time (h)**	**No. of DFOs per antibody**
Vosjan (2010)[10]	Addition of p-SCN-Bn-DFO in DMSO to antibody solution in sodium carbonate buffer (pH 8.9-9.1)	2-10	3	0.1 M Na_2CO_3	8.9-9.1	37°C	0.5	0.3-0.9
Verel (2003)[9]	1. Succinylation of DFO forming N-sucDFO. 2. Complexation with iron to form (Fe^{3+})N-sucDFO. 3. Esterification with tetrafluorophenol (TFP) 4. Reaction with antibody 5. Removal of Fe^{3+} with EDTA	5	~2	0.1 M Na_2CO_3	9.5-9.8	RT	0.5	~1
Meijs (1997)[15]	1. Amine group of DFO modified with SATA to yield thioester. 2. Maleimide groups coupled to the Ab by reaction with SMCC. 3. Deprotection of thiol 4. Coupling of DFO to antibody	1	~10	0.1 M phosphate buffer	6.5	RT	1	1-3
This work	Addition of p-SCN-Bn-DFO in DMSO to antibody solution in sodium bicarbonate buffer (pH 8.9)	0.5	10	0.1 M $NaHCO_3$	8.9	37°C	1	0.85-1.40

^{89}Zr radiolabeling conditions								
Method	**Initial amount of Ab (mg)**	**Means of neutralising/removing oxalic acid**	**Buffer**	**Final pH of reaction mixture**	**Temperature**	**Time (h)**	**Radiolabeling yield % ± SD**	**Specific activity (MBq/µg)**
Vosjan (2010)[10]	0.7-3	2 M Na_2CO_3	0.5 M HEPES (pH 7.1-7.3)	6.8-7.2	RT	1	>85	Not stated
Verel (2003)[9]	5	2 M Na_2CO_3	0.5 M HEPES (pH 7.2-7.4)	7.2-7.4	RT	0.5	80±6	0.01-0.55
Meijs (1997)[15]	0.6-0.75	Oxalic acid removed by sublimation	0.1 M ammonium acetate	Not stated	RT	1	>90	0.185
This work	0.1	1 M Na_2CO_3	PBS (pH 7.4)	7-8	RT	1	96.9±3.3 (n=6)	0.10±0.03
	0.075			7-8			89.8±13.0 (n=6)	0.10±0.02
	0.05			7.5-8			83.4±24.1 (n=6)	0.10±0.03
	0.025			8			84.2±18.1 (n=6)	0.10±0.02
	0.01			8			83.1±21.5 (n=7)	0.10±0.02

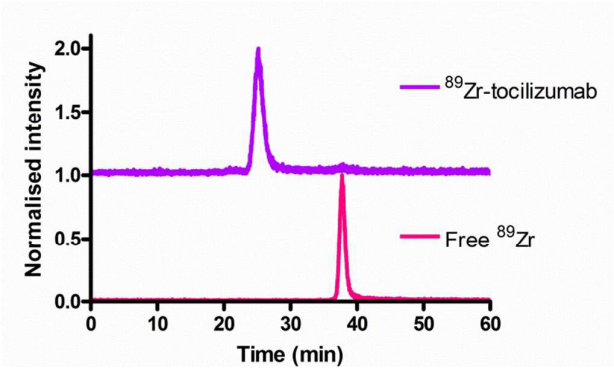

Fig. 3 Radio-HPLC chromatogram of crude reaction mixture using 100 µg of tocilizumab. Retention times: ^{89}Zr-labeled tocilizumab: 25.2 min; Free ^{89}Zr: 37.8 min

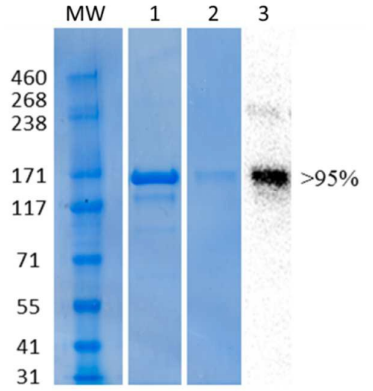

Fig. 4 Representative image of an SDS-PAGE gel performed under non-reducing conditions. Lanes: 1) Unmodified tocilizumab, 2) ^{89}Zr-tocilizumab, 3) Phosphor image of lane 2

incubated at room temperature for 1 h and the radiolabeling efficiency was determined by iTLC using an eluent of 50 mM EDTA (pH 6) and radio-HPLC. The reaction was performed using decreasing amounts of DFO-modified tocilizumab in the range 100 to 10 µg (Table 1). The crude reaction mixture was purified by Sephadex-G50 size exclusion chromatography, eluting with 100 µL fractions of phosphate buffered saline (pH 7.4). After combining the fractions which corresponded to ^{89}Zr-tocilizumab, the radiochemical purity was determined by iTLC and the sample was analysed by SDS-PAGE on a 3-8% tris-acetate gel (NuPAGE, Novex) under non-reducing conditions, followed by Coomassie G-250 staining (SimplyBlue Safestain, Thermo Fisher Scientific) and autoradiography.

^{89}Zr-trastuzumab was prepared in an identical manner using 100 µg of DFO-modified trastuzumab. The immunoreactivity of ^{89}Zr-trastuzumab was determined on MDA-MB-231/H2N cells by linear extrapolation to conditions representing infinite antigen excess according to the method described by Lindmo et al..[16, 17]

RESULTS AND DISCUSSION

Antibody modification with DFO

MALDI-TOF mass spectrometry data and isotopic dilution experiments revealed each tocilizumab molecule was modified with a mean average number of 0.85 and 1.40 DFO chelating groups, respectively (Figs. S1-3). These values are in close agreement with the range of 0.3-0.9 reported by Vosjan et al. in 2010, despite using considerably lower amounts of antibody (500 µg versus 2-10 mg, respectively). Our method differed slightly as a 10-fold molar excess of the bifunctional chelator p-SCN-Bn-DFO was used in the reaction mixture compared to a 3-fold molar excess in the Vosjan protocol and we also allowed a longer reaction time of 1 h.

Antibody radiolabeling reactions with zirconium-89

The results of the tocilizumab radiolabeling reactions are provided in Table 1. Radiolabeling reactions that were performed using 100 µg of DFO-tocilizumab consistently resulted in excellent radiochemical yields (96.9±3.3%) which compare very favourably with the other methods shown in Table 1 which all employ considerably higher quantities of antibody. When performed on a scale of 10-75 µg, high radiochemical yields were also achieved that were typically >80%. While these yields were not significantly lower than those found on the 100 µg scale ($P > 0.05$), a greater variability in results was observed which indicates that the reactions were not as reproducible on this scale. The reactions could be purified to high radiochemical purity (>95%) following either radio-HPLC (Fig. 3) or manual size-exclusion chromatography using G50 Sephadex.

The purified ^{89}Zr-tocilizumab samples were further analysed by SDS-PAGE, alongside unmodified tocilizumab for comparison (Fig. 4). In each case, a single band can be observed corresponding to the molecular weight of tocilizumab (145 kDa). Autoradiography of the gel was also performed which revealed an intense band (>95%) corresponding to ^{89}Zr-tocilizumab at a molecular weight indistinguishable from the Coomassie staining of tocilizumab and ^{89}Zr-tocilizumab. The lack of any degradation products within the lane indicates that the radiolabeled antibody remained intact.

Following the same DFO-modification and radiolabeling procedures, ^{89}Zr-trastuzumab was also synthesised in excellent radiochemical yield (96.5±5.7%, n = 3) using 100 µg of DFO-trastuzumab. An immunoreactive fraction of 0.95±0.07% was determined which indicates a non-significant disruption to the antigen binding properties occurred as a result of the DFO-modification and radiolabeling procedures (Fig. S4).

Conclusions

The method described herein represents a successful miniaturisation of established radiolabeling procedures involving zirconium-89 for immuno-PET. The ability to achieve high radiochemical yields using considerably smaller amounts of antibody compared to the most widely cited methods can provide multiple advantages, particularly during the research and development process. For example, it is likely to reduce the costs associated with purchasing commercial antibodies which can otherwise be prohibitive to a comprehensive and detailed investigation, particularly for the most contemporary antibodies. Additional advantages include the opportunity to use smaller amounts of the zirconium-89 radioisotope which is not only beneficial from a financial perspective but also in terms of reducing radiation dose to the experimenter and thereby adhering to the ALARA principles for radiation safety. Lastly, this approach does not require access to specialised equipment and can be readily implemented in research facilities with minimal radiochemistry infrastructure.

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References

1. M. A. Deri, B. M. Zeglis, L. C. Francesconi and J. S. Lewis, *Nucl. Med. Biol.*, 2013, **40**, 3-14.
2. Y. Zhang, H. Hong and W. Cai, *Curr. Radiopharm.*, 2011, **4**, 131-139.
3. F. C. J. Van De Watering, M. Rijpkema, L. Perk, U. Brinkmann, W. J. G. Oyen and O. C. Boerman, *BioMed Res. Int.*, 2014, **2014**.
4. B. M. Zeglis and J. S. Lewis, *Dalton Trans.*, 2011, **40**, 6168-6195.
5. M. A. Deri, S. Ponnala, B. M. Zeglis, G. Pohl, J. J. Dannenberg, J. S. Lewis and L. C. Francesconi, *J. Med. Chem.*, 2014, **57**, 4849-4860.
6. C. Zhai, D. Summer, C. Rangger, G. M. Franssen, P. Laverman, H. Haas, M. Petrik, R. Haubner and C. Decristoforo, *Mol. Pharm.*, 2015, **12**, 2142-2150.
7. M. Patra, A. Bauman, C. Mari, C. A. Fischer, O. Blacque, D. Haussinger, G. Gasser and T. L. Mindt, *Chem. Commun.*, 2014, **50**, 11523-11525.
8. D. N. Pandya, S. Pailloux, D. Tatum, D. Magda and T. J. Wadas, *Chem. Commun.*, 2015, **51**, 2301-2303.
9. I. Verel, G. W. M. Visser, R. Boellaard, M. Stigter-van Walsum, G. B. Snow and G. A. M. S. van Dongen, *J. Nucl. Med.*, 2003, **44**, 1271-1281.
10. M. J. Vosjan, L. R. Perk, G. W. Visser, M. Budde, P. Jurek, G. E. Kiefer and G. A. van Dongen, *Nat. protoc.*, 2010, **5**, 739-743.
11. L. R. Perk, M. J. W. D. Vosjan, G. W. M. Visser, M. Budde, P. Jurek, G. E. Kiefer and G. A. M. S. Van Dongen, *Eur. J. Nucl. Med. Mol. Imaging*, 2010, **37**, 250-259.
12. B. M. Zeglis and J. S. Lewis, *J. Vis. Exp.*, 2015, e52521.
13. B. M. Zeglis, C. B. Davis, D. Abdel-Atti, S. D. Carlin, A. Chen, R. Aggeler, B. J. Agnew and J. S. Lewis, *Bioconjugate Chem.*, 2014, **25**, 2123-2128.
14. J. P. Holland, V. Divilov, N. H. Bander, P. M. Smith-Jones, S. M. Larson and J. S. Lewis, *J Nucl Med*, 2010, **51**, 1293-1300.

15. W. E. Meijs, H. J. Haisma, R. P. Klok, F. B. Van Gog, E. Kievit, H. M. Pinedo and J. D. M. Herscheid, *J. Nucl. Med.*, 1997, **38**, 112-118.
16. T. Lindmo, E. Boven, F. Cuttitta, J. Fedorko and P. A. Bunn, Jr., *J. Immunol. Meth.*, 1984, **72**, 77-89.
17. T. Lindmo and P. A. Bunn, Jr., *Meth. Enzym.*, 1986, **121**, 678-691.

Graphical Abstract:

A ^{89}Zr-radiolabeling procedure that provides high radiochemical yields at the microgram scale has been reported.