Evaluation of DFO-HOPO as an octadentate chelator for zirconium-89†

L. Allott, ‡a C. Da Pieve, ‡b J. Meyers, ‡b T. Spinks, ‡a D. M. Ciobota, ‡a G. Kramer-Marek ‡a and G. Smith ‡a,‡a

The future of 89Zr-based immuno-PET is reliant upon the development of new chelators with improved stability compared to the currently used deferoxamine (DFO). Herein, we report the evaluation of the octadentate molecule DFO-HOPO (3) as a suitable chelator for 89Zr and a more stable alternative to DFO. The molecule showed good potential for the future development of a DFO-HOPO-based bifunctional chelator (BFC) for the radiolabelling of biomolecules with 89Zr. This work broadens the selection of available chelators for 89Zr in search of improved successors to DFO for clinical 89Zr-immuno-PET.

An increasing interest in zirconium-89 (89Zr) for preclinical and clinical immuno-positron emission tomography (immuno-PET) is due to its favourable decay characteristics (t1/2 = 78.4 h, β+ = 22.2%, Eβ+/max = 901 keV) for the radiolabelling of antibodies which have long biological half-lives.1–4 Currently, deferoxamine (DFO) is the chelator most commonly used to radiolabel biomolecules with 89Zr.5 DFT modelling showed that the coordination sphere in the Zr–DFO complex consists of the Zr4+ cation, six donor atoms belonging to the DFO molecule, and two other coordination sites being occupied by water molecules.6,7 As a result of this incomplete coordination of 89Zr by the hexadentate DFO molecule, the 89Zr–DFO complex undergoes a certain degree of demetallation in vivo with the released 89Zr taken up by the bone.8 This is of concern because bone uptake of free 89Zr4+ is undesirable owing to the high radiation dose to bone marrow; furthermore this background uptake can confound image acquisition of bone malignancies such as bone metastases. To solve the instability, different strategies have been investigated. Alternative hexadentate macrocycles (i.e. Fusaricine C) and hydroxypyridinone-based compounds (i.e. CP256) have been produced and tested but showed either no improvement or reduced stability in vivo when compared to DFO.9,10 Additionally, a variety of either linear or macrocyclic octadentate chelators have been developed with structures hinged around hydroxamic acid or hydroxypyridinone moieties which resulted in 89Zr-complexes displaying either increased or decreased stability compared to DFO.11–19 White et al. described the use of a DFO-1-hydroxy-2-pyridone ligand (DFO-HOPO) as an effective sequestering agent for the treatment of plutonium(v) poisoning (Fig. 1).20 The authors showed that the addition of one 1,2-HOPO molecule to DFO produced a low toxicity octadentate chelator which yielded very stable complexes with Pu(v) at physiological pH. Herein, we report an updated synthesis of DFO-HOPO (3) which was then evaluated as an octahedral ligand for 89Zr. The stability of the radiocomplex was tested in vitro and in vivo and compared to 89Zr–DFO in order to confirm 3 as a viable alternative to the chelators for 89Zr already described in the literature.

The synthesis of DFO-HOPO (3) was adapted from a literature procedure.20 In brief, commercially available DFO was reacted with hydroxamic acid chloride (2) and the product (3) was isolated by semi-preparative RP-HPLC (Scheme S1, ESI†). No protection of the N-hydroxyl group of 1 was necessary. To determine and characterise the coordination capabilities of the chelator, the

![Fig. 1. Structure of DFO–HOFO (3) containing three hydroxamic acid and one hydroxypyridone moiety for coordinating 89Zr4+.](image-url)
non-radioactive natZr complex of DFO-HOPO (natZr-3) was prepared in macroscopic scale by mixing the chelator with ZrCl₄ at room temperature. Showing the value of 784.247 used as a comparison. The elution profiles of both the radioactive values reported in the literature for similar complexes.11,12 distances were in the range of 2.14–2.36 Å, in agreement with eight oxygen atoms of the chelator (Fig. 2). The Zr–O bond energy conformation) shows the metal centre coordinated to were carried out. The optimised geometry (based on the lower octadentate chelate, density functional theory (DFT) calculations were performed due to its poor solubility in any solvent, which is guaranteed a quantitative (99%) radiolabelling up to a specific activity of 20 MBq nmol after 24 hours showed only one product (corresponding to the transition. A comparable radiolabelling efficiency was obtained for ⁸⁹Zr–DFO. All reactions were monitored by radio-active instant thin layer chromatography (radio-ITLC). A variety of mobile and stationary phases were tested to find the optimum analytical conditions for both ⁸⁹Zr–DFO, which was used as a comparison. The elution profiles of both the radioactive complexes were affected by the type of stationary phase employed, and only the positively charged ⁸⁹Zr–DFO (consequence of the hexadentate chelation of ⁸⁹Zr) was influenced also by the mobile phase pH, when SG-ITLC strips were used. The results suggest that, differently from ⁸⁹Zr–DFO, ⁸⁹Zr–3 is present in solution as a neutral complex, achievable through the octadentate chelation of ⁸⁹Zr. This finding further advocates the involvement of the 1,2-HOPO moiety of 3 in the coordination of the metal centre. Enabling the elution of ⁸⁹Zr–3 and the ⁸⁹Zr–DFO as well defined and separated bands (Rᵢ of 0.6 and 0.1 respectively on SG-ITLC strips), ammonium acetate (0.1 M, pH 7) was used as mobile phase for the ITLC analysis. Interestingly, the radio-ITLC of ⁸⁹Zr–3 revealed the presence of two well-separated spots (Rᵢ of ca. 0.6 and 0.1); the relative intensity of the spots was dependent on the specific activity of the product (i.e. concentration of the chelator) and on time. By lowering the specific activities of the product, with a consequent increase of the concentration of 3, a decrease of the band having Rᵢ = 0.1 was observed. After 24 hours at ambient temperature, only the band having Rᵢ = 0.6 was detected. To probe the influence of temperature on the formation of the two products, the radiolabelling reaction was performed at 80 °C. Although the quantity of product eluting with an Rᵢ = 0.1 was reduced, the increased temperature did not prevent it from forming. These observations suggest that the two bands represent two different forms of the ⁸⁹Zr–3 complex; an initial transitional kinetic product which converted into a final thermodynamically stable product. Examination of the chromatographic data of ⁸⁹Zr–3 could help explain the phenomenon; the transitional product was detected at the origin of the radio-ITLC strip (Rᵢ = 0.1 at pH 7) suggesting it was charged (similarly to hexacoordinated ⁸⁹Zr–DFO), possibly as the result of incomplete coordination of the radiometal. With an Rᵢ = 0.6 (at pH 7), the thermodynamically stable final product was most likely neutral, a condition which would be achieved by the complete chelation of ⁸⁹Zr by octadentate 3. Moreover, radio-HPLC analysis of ⁸⁹Zr–3 after 24 hours showed only one product (corresponding to the band with Rᵢ = 0.6 on radio-ITLC) having an elution profile very similar to that of natZr–3 suggesting a similar identity as an octadentate complex. Importantly, no ⁸⁹Zr was released during the transition.

The stability of ⁸⁹Zr–3 was initially assessed by a simple radio-ITLC analysis using an acidic buffer (pH 2) as mobile phase. Differently from ⁸⁹Zr–DFO (14.4 ± 4.65% radioactivity not associated with DFO), ⁸⁹Zr–3 showed no demetallation as a result of the enhanced coordination of the metal centre by the octadentate ligand. To mimic what might happen in vivo, a challenge assay assessed the stability of ⁸⁹Zr–3 to transchelation in the presence of a large excess of either EDTA or DFO (pH 7). In both challenges, ⁸⁹Zr–3 showed no transchelation with >99% intact complex after 7 days (Table 1). By comparison, ⁸⁹Zr–DFO demonstrated transchelation toward EDTA with 65.5% of intact complex after 7 days (Table 1). Moreover, a complete transmetallation of ⁸⁹Zr–DFO towards 3 was achieved in a matter of hours. Further experiments aiming to test the inertness of ⁸⁹Zr–3 were performed in mouse serum. With >99% intact complex after incubation at 37 °C for 7 days, ⁸⁹Zr–3 showed a higher stability compared to ⁸⁹Zr–DFO (90.6% intact complex) (Table 1).

![Fig. 2](https://example.com/fig2.png)

Fig. 2 The DFT optimised structure of ⁸⁹Zr-3 (Atom colour: white = hydrogen; grey = carbon; blue = nitrogen; red = oxygen; cyan = zirconium.)
PET imaging and comparative biodistribution studies were performed in healthy mice for $^{89}\text{Zr}$-3 and $^{89}\text{Zr}$–DFO. At 1 h p.i. of $^{89}\text{Zr}$-3, the radioactivity was observed mainly in the bladder and intestine; some activity was also visible in the gall bladder. At 4 and 24 h p.i., most of the residual radioactivity was in the gut. These observations indicate a rapid renal clearance together with slower hepatobiliary excretion. The hydrophilicity of the complexes is an important physiochemical property which regulates their distribution, metabolism, and elimination in vivo. The log $D_{2,4}$ of neutral complex $^{89}\text{Zr}$-3 was found to be $-0.87 \pm 0.03$ which indicates a less hydrophilic character than the positively charged $^{89}\text{Zr}$–DFO ($-3.0 \pm 0.01$) and can explain the clearance pathway. After 24 h, the radioactivity level was minimal therefore no additional imaging studies at longer time points were carried out. Importantly, no uptake of $^{89}\text{Zr}$ in the bone was observed at any time point (Fig. 3).

Corroborating the PET images, the biodistribution studies clearly showed the participation of both the renal and hepatobiliary systems in the clearance of $^{89}\text{Zr}$-3 (Fig. 4). Most of the radioactivity had already cleared through the kidneys at 1 h p.i. (1.39 ± 0.1% ID per g), while at 4 h p.i. the residual activity was localised in the gut (mostly small intestine with 0.898 ± 0.252% ID per g). Differently from $^{89}\text{Zr}$–DFO (0.93 ± 0.11% ID per g still present in the kidneys), $^{89}\text{Zr}$-3 was almost completely cleared from the body at 24 h p.i. Although the values are quite low, $^{89}\text{Zr}$–DFO showed ca. 10-fold higher activity accumulation in the bone than $^{89}\text{Zr}$-3 at 24 h p.i. (0.037 ± 0.002 and 0.004 ± 0.001 for $^{89}\text{Zr}$–DFO and $^{89}\text{Zr}$-3 respectively). This phenomenon could be correlated to either the higher level of radioactivity still present in the animals injected with $^{89}\text{Zr}$–DFO or to an improved in vivo stability of $^{89}\text{Zr}$-3 compared to $^{89}\text{Zr}$–DFO.

In summary, the $^{89}\text{Zr}$-3 complex exhibited improved stability compared to $^{89}\text{Zr}$–DFO in both challenge assays and in serum; the capability and favourability of 3 to form a stable chelate was clearly demonstrated by the complete transchelation of $^{89}\text{Zr}$ from $^{89}\text{Zr}$–DFO in ca. 3 h. The in vivo studies showed that $^{89}\text{Zr}$-3 cleared the body via the renal and hepatobiliary systems. However, once conjugated to a biomolecule the pharmacokinetics of the final radioconjugate will depend mainly on the biomolecule itself. Importantly, the straightforward synthesis of 3 from the commercially available DFO is amenable to allow the synthesis of a bifunctional chelator which is currently underway in our laboratory. This could be achieved by using a similar strategy described by Patra et al. for the synthesis of DFO*, where a molecule (or a variety of molecules) containing both the bidentate moiety and a reactive functionality for bioconjugation is attached to the free amine of DFO. The promising DFO-HOPO molecule is a valuable addition to the selection of available chelators for $^{89}\text{Zr}$ in search of successful successors of DFO for clinical immuno-PET applications based on important characteristics such as synthesis, chelate stability and in vivo pharmacokinetics.

We thank Tom Burley and Steven Turnock for valuable technical help. This work was supported by the Cancer Research UK – Cancer Imaging Centre (grant ref: C1060/A16464) and Wellcome Trust grant 102361/Z/13/Z. This report is independent research funded by the National Institute for Health Research. The views expressed in this publication are those of the authors.
and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health.

Notes and references