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Radiolabelling of the Octadentate Chelators DFO* and oxoDFO* with Zirconium-89 and Gallium-68

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Abstract

In recent years, clinical imaging with zirconium-89 (^{89}Zr)-labelled monoclonal antibodies (Ab) by positron emission tomography (immunoPET) has been gaining significant importance in nuclear medicine for the diagnosis of different types of cancer. For complexation of the radiometal ^{89}Zr and its attachment to the Ab, chelating agents are required. To date, only the hexadentate chelator desferrioxamine (DFO) is applied in the clinic for this purpose. However, there is increasing preclinical evidence that the ^{89}Zr -DFO complex is not sufficiently stable and partly releases the radiometal *in vivo* due to the incomplete coordination sphere of the metal. This leads to unfavourable unspecific uptake of the osteophilic radiometal in bones, hence decreasing the signal-to-noise-ratio and leading to an increased dose to the patient. In the past, several new chelators with denticities >6 have been published, notably the octadentate DFO derivative DFO*. DFO* however shows limited water solubility, wherefore an oxygen containing analogue, termed oxoDFO*, was developed in 2017. However, no data on the suitability of oxoDFO* for radiolabelling with ^{89}Zr has yet been reported. In this proof-of-concept study, we present the first radiolabelling results of the octadentate, water-soluble chelator oxoDFO*, as well as the *in vitro* stability of the resulting complex ^{89}Zr -oxoDFO* in comparison to the analogous octadentate, but less water soluble derivative DFO* and the current “standard” chelator DFO. In addition, the suitability of DFO* and oxoDFO* for radiolabeling with the short-lived PET metal gallium-68 is discussed.

Keywords: chelators; zirconium-89; gallium-68; positron emission tomography (PET)

Introduction

Radiolabelled antibodies (Ab) have become an indispensable tool in nuclear medicine for the diagnosis (imaging) of tumours due to their excellent specificity towards various antigens important in oncology.[1] In particular, the number of applications of Abs labelled with positron emitting radionuclides (immunoPET) is steadily increasing.[2,3] Several immunoPET agents are currently being evaluated in clinical trials.[4-7] In general, such agents consist of a tumour-targeting monoclonal Ab functionalised with a chelator carrying a positron emitting radiometal.

Abs exhibit slow pharmacokinetics and the time until a sufficient accumulation in the target is achieved, can take days to weeks.[8] In the context of nuclear imaging, this results in the necessity of radionuclides with a matching physical half-life, e.g., zirconium-89 (^{89}Zr , $t_{1/2} = 78.41 \text{ h}$).[9,10] The only chelator currently used in clinical trials utilizing ^{89}Zr -labelled Abs is the siderophore desferrioxamine (DFO **1**, Figure 1). However, the hexadentate chelator DFO **1** is not ideal for the complexation of zirconium since the metal ion mostly has coordination numbers >6 [11,12]. Therefore, a chelator with a higher denticity, e.g., hepta- or octadentate, would be the more optimal choice. It has been suggested that the unsaturated coordination sphere of zirconium in complexes with DFO results in slow release of zirconium *in vivo*. This in turn leads to nonspecific uptake of the osteophilic radiometal in bones, an increased background in the PET image, and an additional unnecessary radiation dose to the patient.[5,13] As a consequence, several octadentate chelating systems for the stable complexation of ^{89}Zr have been reported in the recent past.[14,12,15,16] Several of these new chelating systems utilize 4 hydroxamate moieties for octadentate chelation of the radiometal in order to minimize the transchelation rate *in vivo*. A promising chelator of this series is an octadentate version of DFO, termed DFO* **2** (Figure 1).[17] It has been shown that DFO* **2**

forms more stable complexes with ^{89}Zr than DFO **1** *in vitro* and *in vivo*. In a direct comparison of ^{89}Zr -DFO-trastuzumab with analogous ^{89}Zr -DFO*-trastuzumab in a N87-tumour mouse model, it was shown that the application of DFO* **2** led to significantly less nonspecific uptake of radioactivity in bone structures as well as in the skin, liver, spleen, and ileum.[18] Also, the superiority of DFO* **2** in comparison to DFO **1** and DFO-squaramide[19] for late time point PET imaging was demonstrated in primates utilizing a humanized IgG Ab against the herpes simplex viral protein gD.[20] Based on published preclinical data, it has been concluded that DFO* **2** is a significant improvement to the commonly applied DFO **1**.

A potential limitation of DFO* **2** is represented by its low solubility in water. This in turn could interfere with the conjugation of the chelator to proteins that are affected in their binding and folding behaviour by the presence of higher concentrations of organic co-solvents, such as for example human serum albumin[21] or G10 antibodies[22]. Consequently, three water-soluble versions of DFO* **2** with oxygen-containing backbones were published independently by two groups in 2017 and 2020. Codd and co-workers reported a biosynthetic approach using *Streptomyces pilosus* that provides small amounts of a water-soluble version of DFO* **2** with four oxygen atoms in the ligand scaffold.[23] Three years later, the octadentate ligand DFOB-PPH^NO^CO with two oxygen atoms in the ligand backbone, synthesized from readily available desferrioxamine, was published by the same group.[24] Alternatively, we employed a solid-phase synthesis for the potentially large scale preparation of oxoDFO* **3** (Figure 1). Using the shake-flask-method, $\log D_{7.4}$ values of -1.5 for oxoDFO* **3** and -2.1 for the corresponding zirconium complex $^{\text{nat}}\text{Zr}$ -oxoDFO* **3** were determined.[25] In comparison, the published $\log D$ value for DFO* **2** is $\log D_{7.4} = -0.4397$.[25] Very recently, a theoretical study by Holland was published predicting that oxoDFO* **3** is an excellent candidate for the stable complexation of zirconium with an estimated thermodynamic stability constant of $\log \beta = 54.16$.[26]

Here, we present the first ^{89}Zr -labelling of the water-soluble, octadentate chelator oxoDFO* **3**. In addition, we were interested in exploring the utility of our DFO* and oxoDFO* chelators for the chelation of other radiometals. In that regard, we also report the results of our investigation into the ability of DFO* **2** and oxoDFO* **3** to complex the radiometal gallium-68 (^{68}Ga , $t_{1/2} = 67.7$ min).

Figure 1. Hexadentate DFO **1** and its octadentate analogues DFO* **2** and oxoDFO* **3**.

Experimental Section

Materials and Methods

All chemicals and solvents were obtained from commercial sources and used as received. Millipore water (Milli Q® 8/16 Direct System, Merck) and ethanol (gradient grade $\geq 99\%$, Sigma-Aldrich) were used for HPLC. DFO **1** (mesylate salt) was purchased from Sigma Aldrich. DFO* **2** and oxoDFO* **3** as well as the corresponding ^{89}Zr - and non-radioactive zirconium ($^{\text{nat}}\text{Zr}$)

complexes were synthesized according to previously published methods.[25,17] ^{89}Zr was obtained in 0.1 M oxalic acid as $[\text{}^{89}\text{Zr}]\text{Zr-oxalate}$ ($^{89}\text{Zr-oxalate}$) from Perkin Elmer in medical quality. $[\text{}^{68}\text{Ga}]\text{GaCl}_3$ ($^{68}\text{GaCl}_3$, ca. 1–1.5 GBq) was eluted from an IRE Elit GalleoTM $^{68}\text{Ge}/^{68}\text{Ga}$ generator (Fleurus) with 0.1 M HCl according to the procedure recommended by the manufacturer. The eluate (450–550 MBq in 1.1 mL) was collected in an evacuated vial and used without any further processing.

ESI-MS spectra were recorded with a ThermoFisher Scientific LTQ-Orbitrap Velos. RadioTLC was performed using either iTLC plates (glass fibre, impregnated with silica gel, Varian) or RP-TLC plates (silica gel 60 RP-18 on aluminium, Merck) with the solvent system indicated in the description of individual experiments.[27] RadioTLC images for quantification were obtained using a Packard Instant Imager (measurement time: 1 min).

High performance liquid chromatography (HPLC) was conducted with a Chromaster pre-assembled system equipped with a 5160 pump and a 5140 UV detector (Hitachi / VWR). Radio-HPLC was performed on a Merck Hitachi L-6200A intelligent pump with a Merck Hitachi UV detector L-7400 and a Packard Radiomatic Flo-One Beta detector equipped with a BGO cell for the detection of radioactivity. For all HPLC analyses, a Synergi Fusion column (RP, 4 μ , 80 Å, 30 x 2 mm, Phenomenex) was used under isocratic conditions (5% Ethanol / 20 mM phosphate buffer, pH 7) with a flow rate of 0.4 mL/min and a UV wavelength of 220 nm.

Radiosyntheses

^{89}Zr -labelling reactions were carried out by adapted published methods.[17,18] In brief, stock solutions of the chelators (1 mM) were prepared in water (DFO **1**, oxoDFO* **3**) or DMSO (DFO* **2**). $^{89}\text{Zr-oxalate}$ (20–30 MBq) was transferred to a LoBind Eppendorf® flask and diluted with 0.1 M oxalic acid (pH 1) to a total volume of 200 μL . 90 μL Na_2CO_3 (2 M) solution was added

and the mixture was left standing for 3 min at room temperature (RT, pH 6-7). 2-50 μL of chelator stock solutions (2-50 nmol) was diluted with water to a volume of 200 μL and added to the neutralized solution of ^{89}Zr . HEPES buffer (0.5 M, pH 7.4) was added to a final volume of 1 mL (resulting in final precursor concentrations of 2-50 μM) and the mixture was incubated at RT for up-to 2 h. The progress of the reaction was monitored by iTLC (Figure S2) and RP-TLC (Figure S3) with citrate buffer (0.1 M, pH 4) as mobile phase.

For ^{68}Ga -labelling reactions, 3.3 μL of stock solutions of DFO **1**, DFO* **2** and oxoDFO* **3** (3.3 nmol), were diluted with either 300 μL HEPES buffer (0.5 M, pH 7.4) or HEPES buffer (1.5 M, pH 4.5) in a LoBind Eppendorf® flask, resulting in a final precursor concentration of 10 μM . 20 μL $^{68}\text{GaCl}_3$ (50-200 MBq) were added to each vial. Radiolabelling reactions were performed at RT for 2 h or in a heating block at 95 °C for 10 min. The progress of the reactions was monitored with iTLC and 0.05 M EDTA pH 4.5 as mobile phase. R_f (^{68}Ga -EDTA = 1; ^{68}Ga -**1,2,3** = 0.

Transchelation Experiments

The ^{89}Zr - and ^{68}Ga -complexes of DFO **1**, DFO* **2** and oxoDFO* **3** were prepared with a chelator concentration of 10 μM in HEPES buffer as described above (2 h at RT for ^{89}Zr and 10 min at 95 °C for ^{68}Ga). 10 μL of the solutions were transferred to LoBind Eppendorf® tubes containing 200 μL of solutions of DTPA (5 or 50 mM, corresponding to 10^4 and 10^5 fold excess DTPA, respectively) and gently shaken at RT (final pH 6). RadioTLC (iTLC) of the reaction mixtures were performed at different time points to determine the transchelation of the radiometals to the challenging chelator DTPA (mobile phase for ^{89}Zr : 0.05 M DTPA, pH 5; for ^{68}Ga : 0.05 M EDTA, pH 4.5).[28]

Results and Discussion

Radiolabelling Reactions

First ^{89}Zr -labelling reactions were carried out at different concentrations of oxoDFO* **3** using reported reaction conditions.[29,17] We found that an oxoDFO* **3** concentration of approximately 10 μM resulted in quantitative complexation of ^{89}Zr ($\approx 20\text{--}30\text{ MBq}$) at RT within 2 h (Figure 2B). Similar results have been reported for DFO **1** and DFO* **2**[17] and have been reproduced in this work as well. Importantly, the applied conditions (RT, pH 7.4) are suitable and also used by default for the ^{89}Zr -labeling of proteins, e.g., Abs.[18,29] The chemical identity of the formed complex ^{89}Zr -**3** was confirmed by HPLC comparison with the non-radioactive reference compound $^{\text{nat}}\text{Zr}$ -**3** (Figures 3 and S1).[25] It should be noted that the broad peaks seen in the HPLC trace are due to an isocratic mobile phase. Application of standard reversed phase (RP) HPLC columns failed to separate the compounds, wherefore a special reversed phase/borderline ion exchange (Synergi Fusion, also see Supporting Information) phase had to be used. The broad peaks do not indicate the formation of multiple isomers. The identity of $^{\text{nat}}\text{Zr}$ -**3** was confirmed by LRMS ($m/z_{\text{calculated}} = 912.29\text{ [M+H]}^+$, $m/z_{\text{observed}} = 912.37$; $[\text{M+H}]^+$, Figure S4).

Next, we looked into the radiolabelling kinetics in more detail. Shortly after the start of the reaction, a transient intermediate, which was not further characterized, formed in minor amounts as detected by iTLC (R_f ^{89}Zr -intermediate = 0.2; not observed by RP-TLC). This intermediate converted slowly to the desired radiometal complex (R_f ^{89}Zr -**3** = 0) within 60 min and without release of ^{89}Zr (R_f free $^{89}\text{Zr}^{4+}$ as its citrate = 0.8-1, Figure S3). Deri et al. have reported similar observations during ^{89}Zr -labeling studies of the chelator HOPO.[28] They suggested that the intermediate might correspond to an initially formed kinetic product, which reacts further to the thermodynamically stable final complex.[28] The transient

intermediate formed is rapidly and fully converted to the desired radiolabelled product within 2 h, wherefore its appearance was not considered as a limitation for applications. The clean formation of desired product $^{89}\text{Zr-3}$ with a radiochemical yield (RCY) of $\geq 97\%$ after approximately 90-120 min could be confirmed. Figure 2A shows the formation of $^{89}\text{Zr-3}$ (red curve) as well as the formation of the intermediate (dots) and its conversion to $^{89}\text{Zr-3}$. The radiolabelling efficiency and achievable molar activity (up to 3 GBq/ μmol) was comparable to those of the reported analogous chelator DFO* **2**. [30] Since oxoDFO* **3** was primarily designed for a combination with temperature sensitive proteins, kinetic studies were only performed at room temperature.

Figure 2. A) Formation of $^{89}\text{Zr-3}$ using 10 μM precursor at RT in HEPES (pH 7.4, RT, n = 3); B) RCY for the radiolabelling of oxoDFO* **3** with ^{89}Zr -oxalate at different concentrations of oxoDFO* **3** after a reaction time of 2 h at RT (n=2-3). The blue curve indicates the sum of the intermediate and $^{89}\text{Zr-3}$.

Figure 3. HPLC-chromatograms of oxoDFO* **3**, $^{89}\text{Zr}^{4+}$, $^{\text{nat}}\text{Zr-3}$ and $^{89}\text{Zr-3}$. The identity of $^{\text{nat}}\text{Zr-3}$ (peak at $R_t = 5.9$ min) was also confirmed by mass spectrometry. Slightly different R_t -values is the result of the serial arrangement of the γ - and UV-detectors. UV traces were measured at $\lambda = 220$ nm.

Assessment of the Stability of ^{89}Zr -Complexes

To evaluate the stability of the ^{89}Zr -complexes of DFO **1**, DFO* **2**, and oxoDFO* **3**, they were incubated at RT and pH 6 with an excess of DTPA (5 mM or 50 mM). We chose a slightly acidic pH because ^{89}Zr -DFO has been reported to be more stable under these conditions.[28] The extent of transchelation of ^{89}Zr was determined by TLC at different time points up to 48 h (R_f ^{89}Zr -DTPA = 0.9-1, R_f $^{89}\text{Zr-1,2,3}$ = 0). The experiments performed with 5 mM of DTPA indicated superior stability of $^{89}\text{Zr-2}$ or **3** in comparison to $^{89}\text{Zr-1}$. While $[^{89}\text{Zr}]\text{Zr-DFO}^*$ ($^{89}\text{Zr-2}$) and $^{89}\text{Zr-3}$ were stable in the presence of 5 mM DTPA over a time period of 48 h, 50% of $[^{89}\text{Zr}]\text{Zr-DFO}$ ($^{89}\text{Zr-1}$) converted to $^{89}\text{Zr-DTPA}$ within 24 h (Figure 4). Even though transchelation was observed for all ^{89}Zr -complexes at the higher concentration of DTPA of 50 mM, octadentate DFO* **2** and oxoDFO* **3** clearly formed the more stable complexes with ^{89}Zr than hexadentate DFO **1**. These results are in agreement with previously published data[17] and reflect the

increased stability of Zr-complexes as a result of octadentate coordination of the metal. Furthermore, the challenging experiments at 50 mM allowed for the determination of the stabilities of ^{89}Zr -**2** or **3**, relative to one another. Interestingly, ^{89}Zr -**3** exhibited slightly improved stability at the higher concentration of DTPA than ^{89}Zr -**2** in these experiments. Since the benefit of the improved stability of ^{89}Zr -**2** has already been demonstrated in mice and primates,[18,20] the analogous, water-soluble chelator oxoDFO* **3** is likely to be suitable for *in vivo* applications as well.

Figure 4. Transchelation curves for ^{89}Zr -**1**, **2**, **3** measured in 5 mM and 50 mM DTPA at pH 6 and RT. Data points of ^{89}Zr -**2** (orange line) and ^{89}Zr -**3** (grey line) are overlapping; n = 2-3. These results are additionally summarized in a table, which can be found in the supporting material.

^{68}Ga -Labeling Experiments

Hydroxamate-containing ligands have also found applications in combination with other (PET) radiometals.^[31-33] In particular, complexes of DFO **1** with ^{68}Ga have been used for the labelling of affibodies^[34] and peptides^[35,36] as well as for infection imaging.^[37,38] This is despite the fact that the stability of ^{68}Ga -DFO has been the subject of contradictory discussions.^[35] To further

study the utility of our DFO* **2** and oxoDFO* **3** chelators, we decided to evaluate their suitability as ^{68}Ga chelators. ^{68}Ga -labelling experiments were performed at different pH and temperatures and the stability of the ^{68}Ga -complexes formed were studied. HEPES was chosen as a buffer at two different pH values (pH 4.5 and 7.4) due to its favourable properties to reduce the formation of ^{68}Ga -colloids[39] and in accordance with the ^{68}Ga -radiolabelling conditions used in our clinical routine facilities (pH 4.5) This also allowed us to compare the ^{68}Ga results directly to the radiolabelling results for ^{89}Zr , which were also performed at pH 7.4. In brief, better radiolabelling results were achieved at pH 4.5 than at pH 7.4. For the latter pH, radiolabelling yields were 22-40% for chelators **1**, **2** & **3**. However, complete complexation of $^{68}\text{Ga}^{3+}$ could not be achieved at RT and pH 4.5 within a reasonable reaction time for the short-lived radiometal (Table 1). Unlike in the case of ^{89}Zr , an elevated temperature (95 °C) was necessary to accomplish quantitative ^{68}Ga -radiolabelling. Interestingly, these findings are in contrast with results by Tsionou et al., who reported quantitative ^{68}Ga -radiolabelling of DFO at room temperature within 10 minutes at slightly different pH in ammonium acetate buffer.[40]

Table 1. Radiolabelling of DFO **1**, DFO***2** and oxoDFO* **3** with $^{68}\text{GaCl}_3$ in HEPES at pH 4.5 and stability of the corresponding complexes ^{68}Ga -**1,2,3** (n = 2).

Ligand	T [°C]	t [min]	RCY [%]	Intact complex [%] ^{a)}
DFO 1	95 °C	10	>99	77 ± 1 / 62 ± 1
DFO 1	RT	120	81	-
DFO* 2	95°C	10	98 ± 1	78 ± 3 / 58 ± 2
DFO* 2	RT	120	89 ± 7	-
oxoDFO* 3	95°C	10	>99	51 ± 5 / 37 ± 2
oxoDFO* 3	RT	120	75 ± 5	-

a) Values after incubation for 1 h / 2 h in 5 mM DTPA at pH 6.

The stability of the ^{68}Ga -complexes was assessed by the same transchelation experiments with an excess of DTPA (5 mM) as described above. In general, we found that ^{68}Ga -**1,2,3** were significantly less stable under the applied conditions than the corresponding ^{89}Zr -complexes with a decreasing stability of ^{68}Ga -**1** $\approx$ ^{68}Ga -**2** > ^{68}Ga -**3** (Table 1). Surprisingly, approximately one third of the radiometal was found already transchelated to the challenging chelator DTPA for ^{68}Ga -**1** after 2 h, although ^{68}Ga -complexes of DFO **1** conjugated to peptides and proteins have been reported to be stable *in vitro* and *in vivo*.^[36,35,34,40] Transchelation experiments *in vitro* are appropriate for a relative comparison of the stability of different (radio)metal complexes but only of limited value to predict their stability *in vivo*.

Conclusion

In this proof of concept study, we report the first ^{89}Zr -labelling of the water-soluble, octadentate chelator oxoDFO* **3**. Quantitative complexation of ^{89}Zr -oxalate was achieved at RT and pH 7.4 within 2 h, reaction conditions well-suited for the radiometallation of Abs. The stability of the formed complex ^{89}Zr -**3** as determined *in vitro* by transchelation experiments was found to be similar to that of ^{89}Zr -**2**.^[30] Since the applicability of DFO* **2** for ^{89}Zr -immunoPET has already been demonstrated preclinically,^[20,18] oxoDFO* **3** also represents a promising candidate in particular in cases where the application of co-solvents for conjugation chemistry is an issue due to the sensitivity of the protein of interest.

DFO **1**, DFO* **2**, and oxoDFO* **3** can also be applied for the complexation of ^{68}Ga , however, elevated temperatures are required for quantitative radiolabelling and the formed products ^{68}Ga -**1,2,3** exhibit a lower stability *in vitro* than the corresponding ^{89}Zr -complexes. The

employment of alternatives to commonly used chelators for ^{68}Ga -labeling of biological molecules[40] has been reported to be beneficial in certain cases[35] and thus, further studies are warranted.

In depth investigations towards the application of bifunctional versions of oxoDFO* **3**[25] for the ^{89}Zr -labelling of proteins and antibodies including *in vivo* testing have now become justified by our proof-of-concept study. Efforts to raise funds for the preclinical evaluation of ^{89}Zr -**3** *in vivo* are initiated. In addition, the assessment of the suitability of the novel chelators for complexation of other (radio)metals is currently ongoing and will be reported in due time.

Conflict of interest

Thomas Mindt and Gilles Gasser are co-inventors on pending patent applications describing DFO* and its derivatives. These patent applications are owned by the University of Zurich and the University of Basel. The patent owners have granted an exclusive license to the company ABX Advanced Biochemicals Compounds. The inventors are unrelated to this company.

Supporting information

Additional experimental material (HPLC chromatograms, iTLC pictures, MS spectra, stability data and general notes) is available at <http://doi.xxxxxxxxxxxxxxxxxxxxxxx>

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Graphical abstract

The water-soluble, octadentate chelator oxoDFO* provides stable complexes with the positron emitter Zirconium-89. The radiolabelling can be performed at room temperature and neutral pH and thus, oxoDFO* represents a promising chelator for applications in immunoPET.