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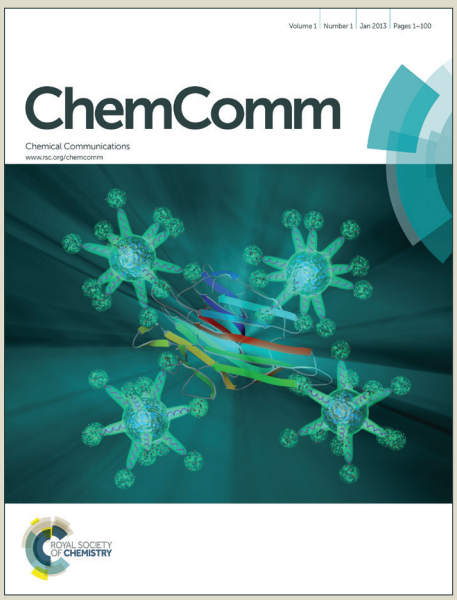

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Di-macrocyclic terephthalamide ligands as chelators for the PET radionuclide zirconium-89†

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The development of bifunctional chelators (BFCs) which can stably chelate zirconium-89 (^{89}Zr) while being conjugated to targeting molecules is an area of active research. Herein we report the first octadentate terephthalamide ligands, which are easily radiolabeled with ^{89}Zr and are highly stable *in vitro*. They represent a novel class of chelators, which are worthy of further development as BFCs for ^{89}Zr.

Zirconium-89 (^{89}Zr: $t_{½}$ = 78.4 h, β^+: 22.8 %, $E_{\beta+max}$ = 901 keV; EC: 77%, E_γ = 909 keV; 99%) has received considerable interest as a positron-emitting radionuclide due to its standardized production, long half-life of 3.3 days, favorable decay characteristics for PET imaging and its successful use in a variety of clinical and preclinical applications.[1,2] However, its successful use in these applications relies upon bifunctional chelators (BFCs) which can stably chelate zirconium-89 while being conjugated to targeting molecules necessary for PET imaging. Unfortunately, Zr's high charge (+4) small radius (59-89 pm for coordination number, CN, 4-9), and the limited data concerning Zr^{4+} coordination chemistry have retarded progress in ^{89}Zr BFC development.[1]

Currently, the most successfully used ^{89}Zr chelator is desferrioxamine B (DFO), which is commercially available as the iron chelator *Desferal* (DF), a microorganism-produced siderophore bearing 3 hydroxamate groups.[3] Although a crystal structure of Zr-DFO has not been reported, DFT modelling of the metal complex suggests that it is eight coordinate with two coordination sites on the Zr^{4+} ion occupied by water molecules.[2f, 4] However, despite the wide acceptance of ^{89}Zr-DFO in PET applications, there is debate regarding the stability of this radiometal complex *in vivo*.[2f] In order to overcome the potential issues of transchelation and non-specific accumulation in tissues, several studies have focused on modifying the conjugation chemistry needed to link DFO to antibodies or developing more effective chelators.[5]

Our research focused on preparing the BFCs **1** and **2** containing terephthalamide (TAM) coordinating units to form an eight coordinate complex to bind the Zr^{4+} cation with greater avidity (**Figure 1**).[6] Moreover, ligand properties such as charge and solubility can be more easily modified using TAM units relative to other chelating units described in the literature.[6c] Additionally, unlike previously reported ^{89}Zr chelators, we incorporated into our ligand scaffolds a pendant arm containing a primary amine, which can be easily functionalized for conjugation to a variety of targeting ligands. These ligands were prepared by condensation of tetraamine and activated di-acid intermediates under high dilution (H.D.) conditions, resulting in the generation of two distinct regioisomers that were separated by chromatography and elaborated into **1** and **2**. Further details of the synthesis are provided in the Supporting Information (SI). The nonradioactive ^{Nat}Zr-**1** and ^{Nat}Zr-**2** complexes were prepared by reacting ligands (**1** and **2**, 1 equiv. each) with a slight excess of $ZrCl_4$ (1.5 equiv.) in water under neutral conditions for 1 h at room temperature. ESI-MS analysis of ^{Nat}Zr-**1** and ^{Nat}Zr-**2** complexes confirmed the 1:1 binding of Zr^{4+} and ligands (**1** and **2**) (See SI).

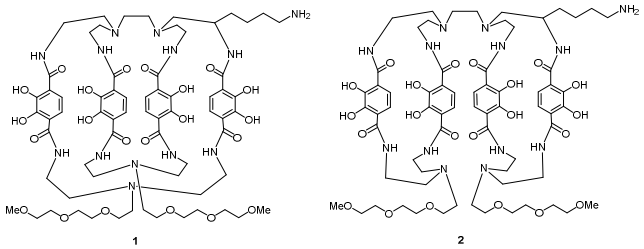

Figure 1. BFCs **1** and **2**. Each di-macrocyclic terephthalamide ligand contains 8 anionic oxygen donor atoms for efficient coordination of the Zr^{4+} ion.

Ground state density functional theory (DFT) calculations were performed for [Zr-**1**]$^{4-}$ and [Zr-**2**]$^{4-}$ using Gaussian 09 (See SI).[7] The structures of the two complexes appear strikingly similar despite the differences in the connectivity of the ligands. The minimized structure of [Zr-**1**]$^{4-}$ was found to be 2.2 kcal/mol lower in energy than the structure of [Zr-**2**]$^{4-}$, a small difference given the size and flexibility of the ligands. In both structures the coordination environment of the Zr^{4+} ion is closest to an *llll*-edge antiprism, with approximate D_4 site symmetry.

Radiochemistry studies demonstrate that all ligands were quantitatively radiolabeled within 15 minutes at ambient temperature (**Table 1**); as good as the best radiochemistry conditions described for recently published ^{89}Zr chelators.[5a-c] Specific activities (A_s) for each ^{89}Zr-**1** and ^{89}Zr-**2** were 997 MBq/μmol and 985 MBq/μmol respectively, and are in accord with the A_s of other ^{89}Zr complexes reported in the literature.[3, 5a-c]

Lipophilicity (log P), which is a fundamental physiochemical property that plays a pivotal role in the adsorption, distribution, metabolism, and elimination of ^{89}Zr-complexes *in vivo*, was determined using a water/octanol partition.[8] Based upon the results of these studies, all complexes demonstrate hydrophilic character,

which is most likely due to both charge (expected to be −3 at neutral pH vs. +2 for ^{89}Zr-**DFO**) and the numerous hydrogen bonding motifs these ligands present in solution. This might suggest that renal excretion would be a preferred route of elimination after *in vivo* injection (**Table 2**).

Table 1. Summary of optimized radiochemistry conditions

Ligand	DFO	1	2
Quantity (μg)	10	10	10
T(ºC)	24	24	24
Reaction Time (min)	15	15	15
Reaction pH	7-7.5	7-7.5	7-7.5
Radiochemical Yield (%)	100	100	100
Specific Activity (A_s; MBq/μmol)	1005	997	985

Table 2. Log P values for all ^{89}Zr-complexes

Complex	log P (n = 12)
^{89}Zr-**DFO**	-2.83±0.04
^{89}Zr-**1**	-3.38±0.04
^{89}Zr-**2**	-3.38±0.03

The stability of each ^{89}Zr-complex was evaluated *in vitro* by incubation at 37ºC in a buffered 50 mM DTPA solution (**Table 3**) and human serum for seven days (**Table 4**). ^{89}Zr-**1** and ^{89}Zr-**2** were more resistant to DTPA challenge than ^{89}Zr-**DFO** over the seven-day study. Additionally, ^{89}Zr-**1** and ^{89}Zr-**2** displayed comparable stability to ^{89}Zr-**DFO** in serum during the study, with no protein transchelation occurring for these complexes. These superior characteristics are believed to result from the ability of the TAM ligands to coordinate the oxophilic Zr^{4+} ion in an octa-coordinated fashion using the 8 anionic oxygen donor atoms in the ligand architecture.[5c] Further studies such as acid and metal ion titration experiments, ligand competition binding assays, and single crystal x-ray crystallography will provide further insight into the mechanism of zirconium complexation and are currently underway in our laboratories.

Table 3. Stability of ^{89}Zr-complexes in 50 mM DTPA (pH 7)

Day	^{89}Zr-DFO (%)[9]	^{89}Zr-1 (%)[9]	^{89}Zr-2 (%)[9]
1	55	100	100
2	54	100	100
3	53	100	100
4	47	100	100
5	44	100	100
6	43	100	100
7	41	100	100

Table 4. Stability of ^{89}Zr-complexes in human serum

Day	^{89}Zr-DFO (%)[9]	^{89}Zr-1 (%)[9]	^{89}Zr-2 (%)[9]
1	100	100	100
2	100	100	100
3	100	100	100
4	100	100	100
5	100	100	100
6	100	100	100
7	100	100	100

The biodistributions of ^{89}Zr-**1** and ^{89}Zr-**2** were determined in normal mice (See SI.). Clearance of ^{89}Zr-**1** from all tissues occurred more rapidly when compared to ^{89}Zr-**2** at every time point. For example, ^{89}Zr-**1** demonstrated faster clearance from the blood, liver, kidney, and bone compared to ^{89}Zr-**2** even at 72 h post-injection (^{89}Zr-**1** vs ^{89}Zr-**2**: %ID/g ± SD) (blood, 0.002±0.002 vs. 0.004±0.002; liver, 0.38±0.08 vs. 0.95±0.08; kidney, 4.77±0.76 vs. 24.38±8.64; bone, 0.07±0.02 vs 0.25±0.03). While both ligands are structurally similar, ^{89}Zr-**1** might bind to plasma proteins less well than ^{89}Zr-**2**. This difference may relate to the different macrocyclic systems present in the structural isomers, which are comprised of two 26-

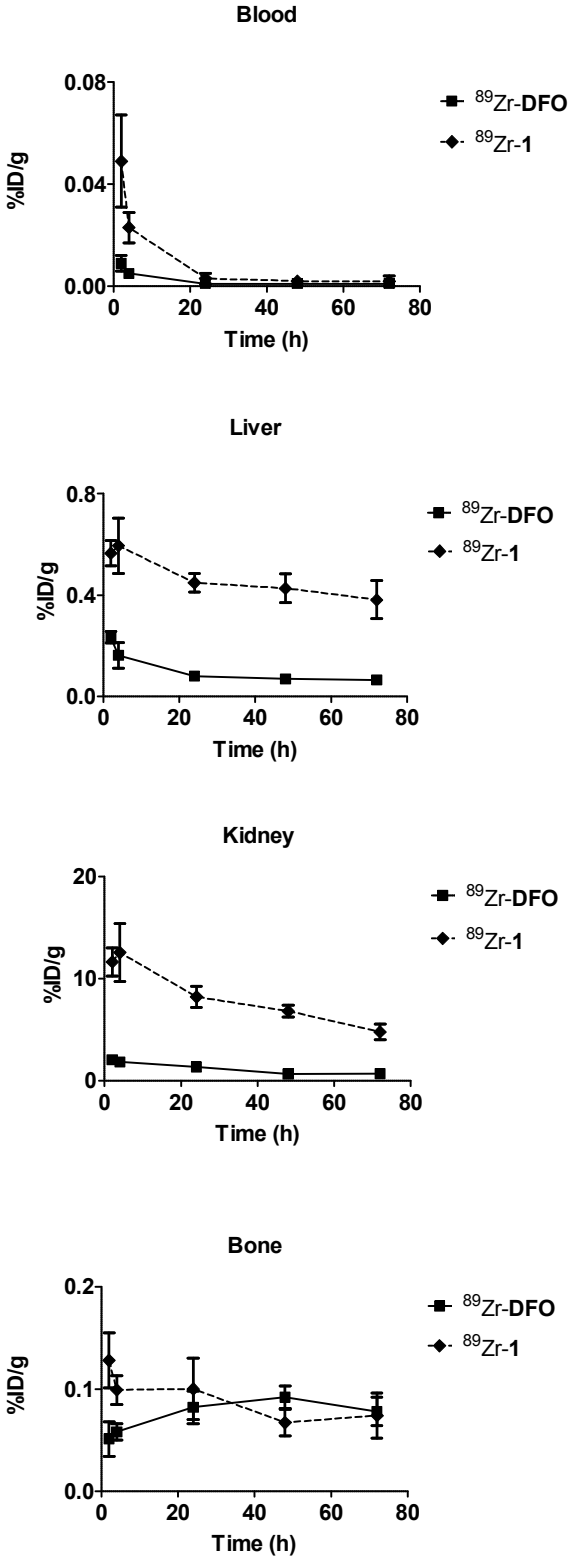

Figure 2. Biodistribution and clearance of ^{89}Zr-**DFO** and ^{89}Zr-**1** from selected tissues (n = 6 for both cohorts).

atom rings in the case of **2**, and two 29 atom rings in the case of **1** (**Figure 1**). Alternatively, the greater flexibility in **2** may allow for the dissociation of a single macrocycle that might leave the Zr^{4+} ion exposed and vulnerable to attack by endogenous (protein) ligands, resulting in its decreased *in vivo* stability compared to ^{89}Zr-**1**.

Figure 2 displays the clearance properties of ^{89}Zr-**1** and ^{89}Zr-**DFO** from the blood, liver, kidney, and bone. Compared to ^{89}Zr-**DFO**, ^{89}Zr-**1** demonstrated comparable blood retention at 72 h post-injection (phi.) (^{89}Zr-**DFO** vs ^{89}Zr-**1**: %ID/g ± SD) (blood, 0.000±0.001 vs. 0.002±0.002). However, liver and kidney retention remained elevated (^{89}Zr-**DFO** vs ^{89}Zr-**1**: %ID/g ± SD) (liver, 0.07±0.01 vs. 0.38±0.08; kidney, 0.69±0.09 vs. 4.77±0.76). Increased retention of activity in the liver may result from aggregation while the increased retention of activity in the kidney may be a more complex, multi-factorial phenomenon. Although aggregation cannot be ruled out, the increasingly acidic environment within the kidney may induce changes to the molecular structure or charge of ^{89}Zr-**1** causing it to be retained with this organ. While augmentation and derivatization of the incorporated PEG groups may allow us to enhance kidney clearance, antibody attachment will cause the greatest changes to chelator biodistribution since it will be supplanted by that of the resulting bioconjugate. This will ultimately determine the utility of this ligand as a BFC for ^{89}Zr.

Given the affinity of ^{89}Zr for the phosphate-rich environment of hydroxyapatite, reducing ^{89}Zr in bone through effective chelation and clearance is an important criterion for new BFCs for ^{89}Zr. We observed that the amount of radioactivity retained in the bones of animals receiving ^{89}Zr-**1**, versus those injected with ^{89}Zr-**DFO**, was not significantly different (^{89}Zr-**DFO** vs ^{89}Zr-**1**: %ID/g ± SD; p value) (bone 0.078±0.014 vs. 0.074±0.022; p = 0.6). Furthermore, ^{89}Zr-**1** demonstrated less bone retention than other ^{89}Zr-systems recently reported in the literature, and we speculate that this similarity to ^{89}Zr-**DFO** was a matter of effective and stable chelation in addition to efficient perfusion.[5c]

In summary, we report two new di-macrocyclic terephthalamide ligands that efficiently complex ^{89}Zr *in vitro*. ^{89}Zr-**1** was cleared more rapidly *in vivo* compared to ^{89}Zr-**2**. The amount of radioactivity retained in the bones of animals receiving either ^{89}Zr-**1** or ^{89}Zr-**DFO** was comparable. These data suggest that chelator **1** might be the preferred choice for further development. This work extends the inventory of chelators available for ^{89}Zr and advances this field through the creation of a ligand system which chelates ^{89}Zr rapidly and has the potential to be easily conjugated to biomolecules.

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Electronic Supplementary Information (ESI) available: synthesis, characterization, biodistribution and experimental details. See DOI: 10.1039/c000000x/

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†Electronic supplementary information (ESI) available: Syntheses, characterization, complete biodistribution and experimental details.

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