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PAPER

Structure-related variable responses of calcium sensitive MRI probes

Ilgar Mamedov,[a] Nikos K. Logothetis[a,b] and Goran Angelovski*[a]

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A new series of Gd^{3+} complexes based on DO3A (**GdL[1]**–**GdL[4]**) was synthesized and investigated. They possess side chains with different structures which determine their varying binding properties and response towards endogenous metal ions, measured by changes in the longitudinal relaxivity (r_1). **GdL[4]** exhibits the highest selectivity toward Ca^{2+} in comparison to the other complexes, with up to a 63% increase of the r_1. **GdL[2]** and **GdL[3]** also respond to different Ca^{2+} concentration ranges, however with a lower selectivity since the r_1 changes are also observed in the presence of other cations such as Mg^{2+}, Zn^{2+} or Cu^{2+}. Assessment of the hydration number (q) *via* luminescence lifetime measurements confirmed that the change in q is responsible for the r_1 response for all the complexes.

Introduction

The use of Gd^{3+} based contrast agents for magnetic resonance imaging (MRI) has enhanced the potential of this technique and its application in clinical trials and biological research.[1] Recent developments in the chemistry of contrast agents have focused on the design of new categories which improve the contrast observed in the MR image.[2] One category, responsive or smart MR agents (SCAs), has become very important in this field of molecular imaging. These agents are designed to follow extra- or intracellular activities *in vivo*, and they are tailored in such a way to increase or decrease the MR signal intensity only in the presence of a given ion or molecule or upon the occurrence of a specific event. The choice of the biochemical target, the chelating moiety and the physiological/pathological environments are critical deterministic parameters for the design, development and application of SCAs in imaging the biological functions.

Several extensive studies on metal sensitive SCAs have been reported recently and are the topic of extensive review articles.[3,4] The alteration of the MR signal intensity in these molecules occurs through structural changes induced by a fluctuation in the concentration of a specific metal ion (*e.g.* Na^+, K^+, Ca^{2+}, Fe^{2+}/Fe^{3+}, Cu^{2+} or Zn^{2+}) that has a critical functional role in organisms. Methods that enable the detection of concentration changes of such molecules *in vivo* have therefore become of great importance over the last few years.[5,6] For example, developments of responsive biochemical functional markers that detect neuronal activity promise direct visualization of neural activation, with a spatiotemporal resolution and specificity that is likely to be much greater than that afforded by hemodynamics.[7] Specifically, extracellular Ca^{2+} acts as "secondary messenger" and plays an important role in regulating a variety of neuronal processes. The ability to non-invasively observe its fluctuations in concentration would be of obvious importance for understanding brain function.

To this end various structures, mostly Gd^{3+}–DO3A (1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid) macrocyclic moieties, have been combined with acyclic chelators that can form complexes with the target metal ion, Ca^{2+}. A series of such complexes which can act as a Ca^{2+} sensors were developed in our group.[8–10] These studies show that the proper choice of the Ca^{2+} chelator and binding mode are crucial to achieve the desired selectivity of the chelators towards Ca^{2+} against competing cations such as Mg^{2+}, Zn^{2+} or Cu^{2+}. Similarly, other groups that focused on the development of SCA sensitive to Zn^{2+} or Cu^{2+}, concluded that even a minor change in the structure of the ligand could have a dramatic influence on the selectivity and sensitivity of the contrast agents towards the target metal ion.[11,12]

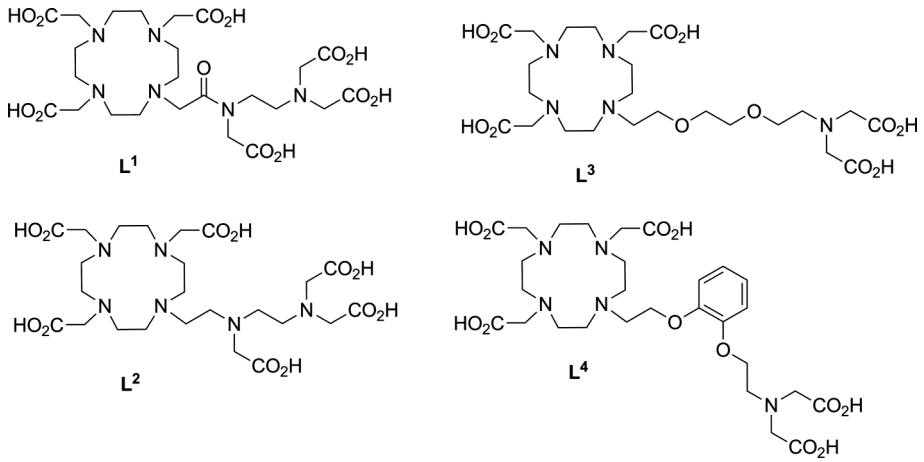
Consequently, an investigation that explores the different types of Ca^{2+} chelators and the various coupling modes to the paramagnetic centre, would be most useful in furthering the progress of Ca^{2+} sensitive SCA. Here we report four Gd^{3+}–DO3A based molecules that bear polyaminocarboxylates and polyetheraminocarboxylates as Ca^{2+} chelators. These two units are conjugated with side chains of varying length and rigidity (Scheme 1). Their synthesis and relaxivities as a function of added endogenous cations and chelator type are investigated and reported.

Results and discussion

Synthesis of ligands and complexes

Several polyaminocarboxylates, such as EDTA (2-[2-[bis(carboxymethyl)amino]ethyl-(carboxymethyl)amino]acetic acid), EGTA (ethyleneglycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) and pyro-EGTA (1,2-bis(2-aminoethoxy)benzene-*N*,*N*,*N*′,*N*′-tetraacetic acid) are known to be very efficient Ca^{2+}

[a]*Department for Physiology of Cognitive Processes, Max Planck Institute for Biological Cybernetics, Spemannstr. 38, 72076, Tübingen, Germany. E-mail: goran.angelovski@tuebingen.mpg.de; Fax: +49 7071 601 919; Tel: +49 7071 601 916*
[b]*Imaging Science and Biomedical Engineering, University of Manchester, Manchester, UK*



Scheme 1 The structures of studied ligand $\mathbf{L}^{1-4}$.

chelators as they form complexes with $\log K_{CaL} \approx 10$.[13] However, in order to couple these types of chelators to a DO3A reporting unit, a derivative of these chelators must be used where specific functional groups are excluded or replaced to enable the coupling of this type of chelator to the DO3A moiety.[8–10] Furthermore, an alteration of the EDTA, EGTA or pyro-EGTA is required to reduce the affinity of the chelator towards Ca^{2+}, making it more sensitive to extracellular Ca^{2+} concentration ranges (low mM).[14] Unfortunately, an intentional decrease in the binding affinity and simplification of the synthesis can lead to a loss of selectivity towards Ca^{2+} over other ions, or a weaker relaxometric response due to a lower number of functional groups that can both interact with Ca^{2+} and Gd^{3+}.

Four different ligands were prepared, and in each case a unique synthetic strategy (A–D, Scheme 2) was applied for their preparation, to allow each side chain to be effectively coupled to the macrocyclic moiety.

The ligands were synthesized in a series of multi-step reactions commencing with the commonly used precursor molecules tris-*t*Bu-DO3A[15] (tri-*tert*-butyl 1,4,7,10-tetraazacyclododecane-1,4,7-triacetate) for the synthesis of $\mathbf{L}^1$, $\mathbf{L}^3$ and $\mathbf{L}^4$, and tris-Boc-cyclen[16] (1,4,7-tris-*tert*-butoxycarbonyl-1,4,7,10-tetraazacyclododecane) for the synthesis of $\mathbf{L}^2$.

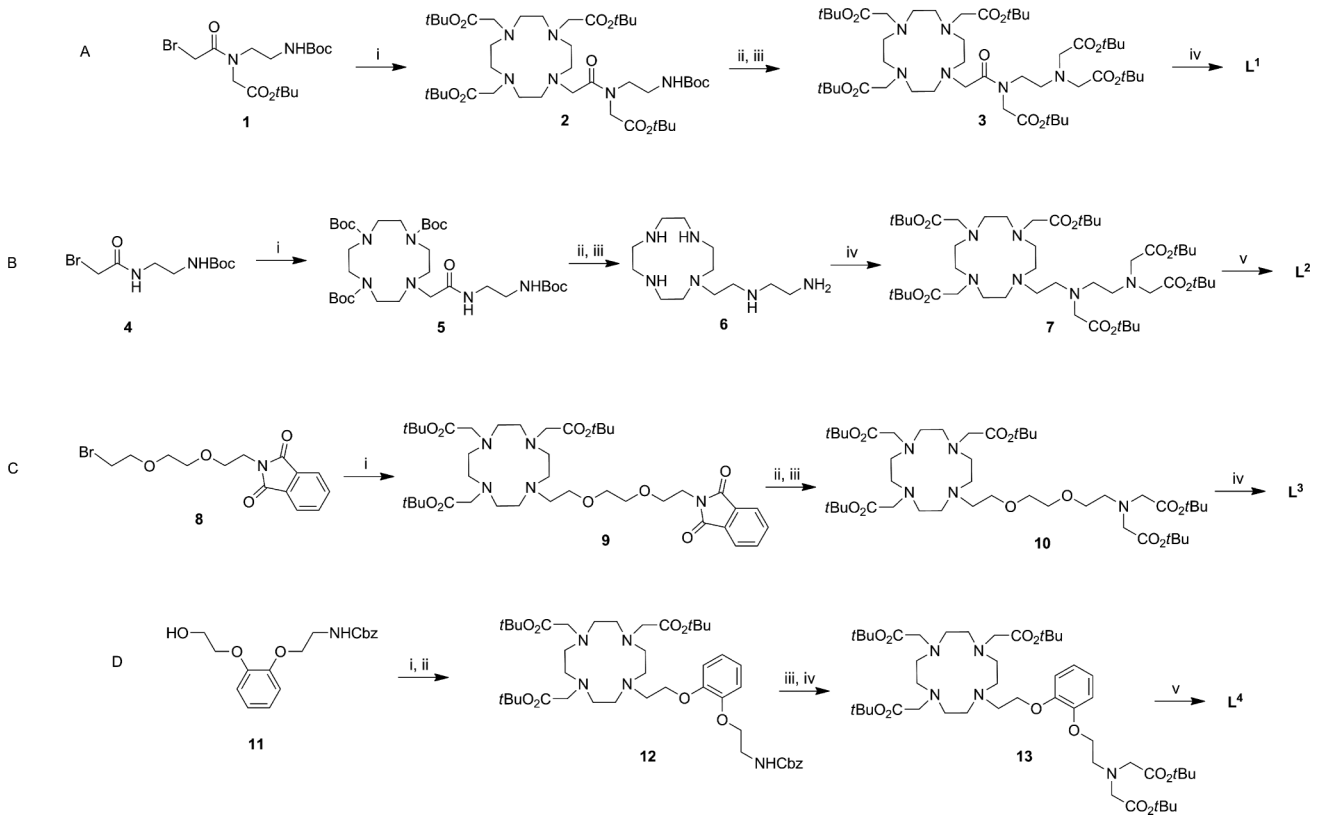
The synthesis of ligand $\mathbf{L}^1$ started with the bromide **1**[17] (Scheme 2A). The alkylation of tris-*t*Bu-DO3A resulted in the macrocycle **2**. After the selective deprotection of the Boc protected amino groups using 2 M HCl solution in MeOH at 0 °C and further alkylation of the obtained product with the *tert*-butyl bromoacetate, the resulting hexaester **3** was isolated. Hydrolysis of the *tert*-butyl esters in the presence of TFA and precipitation from methanol/diethyl ether yielded the final ligand $\mathbf{L}^1$ as a colorless hygroscopic powder.

$\mathbf{L}^2$ was synthesized by the alkylation of tris-Boc-cyclen with **4**,[18] in MeCN at 60 °C using K_3CO_3 as a base (Scheme 2B). After the complete deprotection of the Boc protected amines using 2 M HCl solution in MeOH, the amide bond in this intermediate product was reduced with $LiAlH_4$ in THF, resulting in **6**. The hexaamine was alkylated with the *tert*-butyl bromoacetate to give the hexaester **7**. After the hydrolysis of the *tert*-butyl esters using formic acid and recrystallization of the product from water/acetone, the final ligand $\mathbf{L}^2$ was obtained as a colorless hygroscopic powder.

The first precursor **8** for the synthesis of $\mathbf{L}^3$ was prepared according to a literature procedure.[19] The alkylation of tris-*t*Bu-DO3A with **8** yielded the macrocycle **9** (Scheme 2C). Cleavage of the phthalimide was achieved by refluxing **9** with a solution of ethylenediamine in isopropanol. The alkylation of resulting amine with *tert*-butyl bromoacetate yielded **10**. The hydrolysis of the *tert*-butyl esters with TFA resulted in $\mathbf{L}^3$ as a yellow oil. All attempts to recrystallize $\mathbf{L}^3$ failed due to its high hygroscopicity. The product was therefore purified by reverse phase HPLC and upon the evaporation of the solvents, $\mathbf{L}^3$ was obtained as a yellow hygroscopic powder.

The first molecule in the synthetic route of $\mathbf{L}^4$ was the aryl-containing compound **11** (Scheme 2D). It was obtained through the alkylation of the commercially available 2-(2-hydroxyethoxy)phenol with *N*-benzyloxycarbonyl-2-bromoethylamine, according to a literature procedure.[20] The alcohol in **11** was brominated in a biphasic solution of thionyl bromide in cyclohexane and the resulting product was used in the alkylation of tris-*t*Bu-DO3A to give **12**. Reductive cleavage of the Cbz protective group by catalytic hydrogenation and further alkylation of this intermediate product with *tert*-butyl bromoacetate yielded **13**. The final ligand $\mathbf{L}^4$ was obtained after the hydrolysis of the *tert*-butyl esters with formic acid, followed by recrystallization from water/acetone, as a brown hygroscopic powder.

The corresponding Gd^{3+} and Eu^{3+} complexes of $\mathbf{L}^1$–$\mathbf{L}^4$ were formed by mixing stock solutions of the metal chlorides with the corresponding ligands in a 1 : 1 ratio. The solutions were heated to 60 °C for 24 h while the pH was maintained at ~7. The complexes were finally treated with Chelex 100 to remove the excess of the lanthanide ions, and the resulting solutions were filtered and lyophilized. The xylenol orange test was performed to ensure no free metal ions were present in solution.[21] The formation of the complexes was confirmed by ESI-MS in negative and positive modes. All the analyzed spectra contained the appropriate molecular ion peaks with the isotope pattern characteristic of the Gd^{3+} and Eu^{3+} complexes.



Scheme 2 Synthesis of the ligands $\mathbf{L^{1-4}}$. *Reagents and conditions*: A: (i) tris-*t*Bu-DO3A, K_2CO_3, MeCN, 70 °C, 12 h; (ii) 2 M HCl in MeOH, 0 °C, 30 min; (iii) *tert*-butyl bromoacetate, K_2CO_3, MeCN, 70 °C, 12 h; (iv) TFA, CH_2Cl_2, 12 h. B: (i) tris-Boc-cyclen, K_2CO_3, MeCN, 70 °C, 18 h; (ii) 2 M HCl in MeOH, 2 h; (iii) $LiAlH_4$, THF, reflux, 18 h; (iv) *tert*-butyl bromoacetate, K_2CO_3, MeCN, 70 °C, 18 h; (v) HCOOH, 60 °C, 12 h. C: (i) tris-*t*Bu-DO3A, K_2CO_3, MeCN, 70 °C, 18 h; (ii) EDA in *i*-PrOH, reflux, 24 h; (iii) *tert*-butyl bromoacetate, K_2CO_3, MeCN, 60 °C, 12 h; (iv) TFA, CH_2Cl_2, 12 h. D: (i) thionyl bromide, cyclohexane, DMF, rt; (ii) tris-*t*Bu-DO3A, K_2CO_3, MeCN, 70 °C, 12 h; (iii) H_2, Pd/C, MeOH, 12 h; (iv) *tert*-butyl bromoacetate, K_2CO_3, MeCN, 18 h; (v) HCOOH, 60 °C, 12 h.

Relaxivity experiments

Relaxivity titrations with Ca^{2+} were performed on $\mathbf{GdL^1}$–$\mathbf{GdL^4}$ (~2.5 mM) at a magnetic field of 7 T and 25 °C and at neutral pH (HEPES buffer). The longitudinal relaxivity, r_1, was calculated from eqn (1), where $T_{1,obs}$ is the measured T_1, $T_{1,d}$ is the diamagnetic contribution of the solvent and [Gd] is the concentration of the appropriate Gd^{3+} complex.

$$\frac{1}{T_{1,\mathrm{obs}}} = \frac{1}{T_{1,\mathbf{d}}} + r_1 \times [\mathrm{Gd}] \qquad (1)$$

The initial r_1 values were found to be similar for three of the complexes (4.44, 4.35 and 4.42 $mM^{-1}s^{-1}$ for $\mathbf{GdL^1}$, $\mathbf{GdL^3}$ and $\mathbf{GdL^4}$ respectively) whilst a slightly lower r_1 value of 3.67 $mM^{-1}s^{-1}$ was found for $\mathbf{GdL^2}$. The addition of Ca^{2+} to the buffered solutions of $\mathbf{GdL^1}$–$\mathbf{GdL^4}$ revealed the very different relaxometric responses of these complexes (Fig. 1). $\mathbf{GdL^1}$ has a very weak response upon its saturation with Ca^{2+} which does not exceed 10% when compared to the initial r_1 value. The r_1 relaxivity of $\mathbf{GdL^2}$ is saturated upon the addition of less than 3 equiv. of Ca^{2+} and increases to 55% above the initial r_1 value. Similarly, the addition of a comparable amount of Ca^{2+} increases the r_1 of $\mathbf{GdL^3}$ to 30%. However, the highest increase in r_1 (63%) is observed for $\mathbf{GdL^4}$ which is saturated with the addition of almost 5 equiv. of Ca^{2+}. The fitting of the Ca^{2+} titration curves resulted in the apparent association constants of $\log K_A$ = 3.1 ± 0.4, 3.4 ± 0.5 and 2.2 ± 0.1 for $\mathbf{GdL^2}$, $\mathbf{GdL^3}$ and $\mathbf{GdL^4}$, respectively, which demonstrates the sensitivity of the investigated complexes towards Ca^{2+} in the desired, low millimolar range, observed in the extracellular region *in vivo*.

The results from the relaxometric titration reveal how a change in the overall structure can produce a significantly different MR response towards Ca^{2+}. To further investigate their behaviour and obtain a better understanding on the responses of these complexes towards other endogenous metal ions (Zn^{2+}, Cu^{2+}, Na^+, K^+, Mg^{2+}), we performed a relaxometric screening where the r_1 values of $\mathbf{GdL^1}$–$\mathbf{GdL^4}$ were determined upon the addition of at least one equivalent of metal ion, before and after the addition of an amount of Ca^{2+} (that causes the maximal change in r_1 for each complex, Fig. 2).

$\mathbf{GdL^1}$ was found to be unresponsive to the presence of any of the above mentioned cations, with its relaxivity remaining almost constant. This behaviour is expected, since the amide group in vicinity of DO3A moiety will most probably coordinate to the lanthanide ion, preventing any type of interaction/coordination of the additional functional groups. The relaxivity enhancement for $\mathbf{GdL^2}$ was observed almost to the same extent upon the addition of Mg^{2+} and Zn^{2+} (45% and 67% increase in r_1, respectively) as seen on the addition of Ca^{2+}. This clearly demonstrates the low selectivity

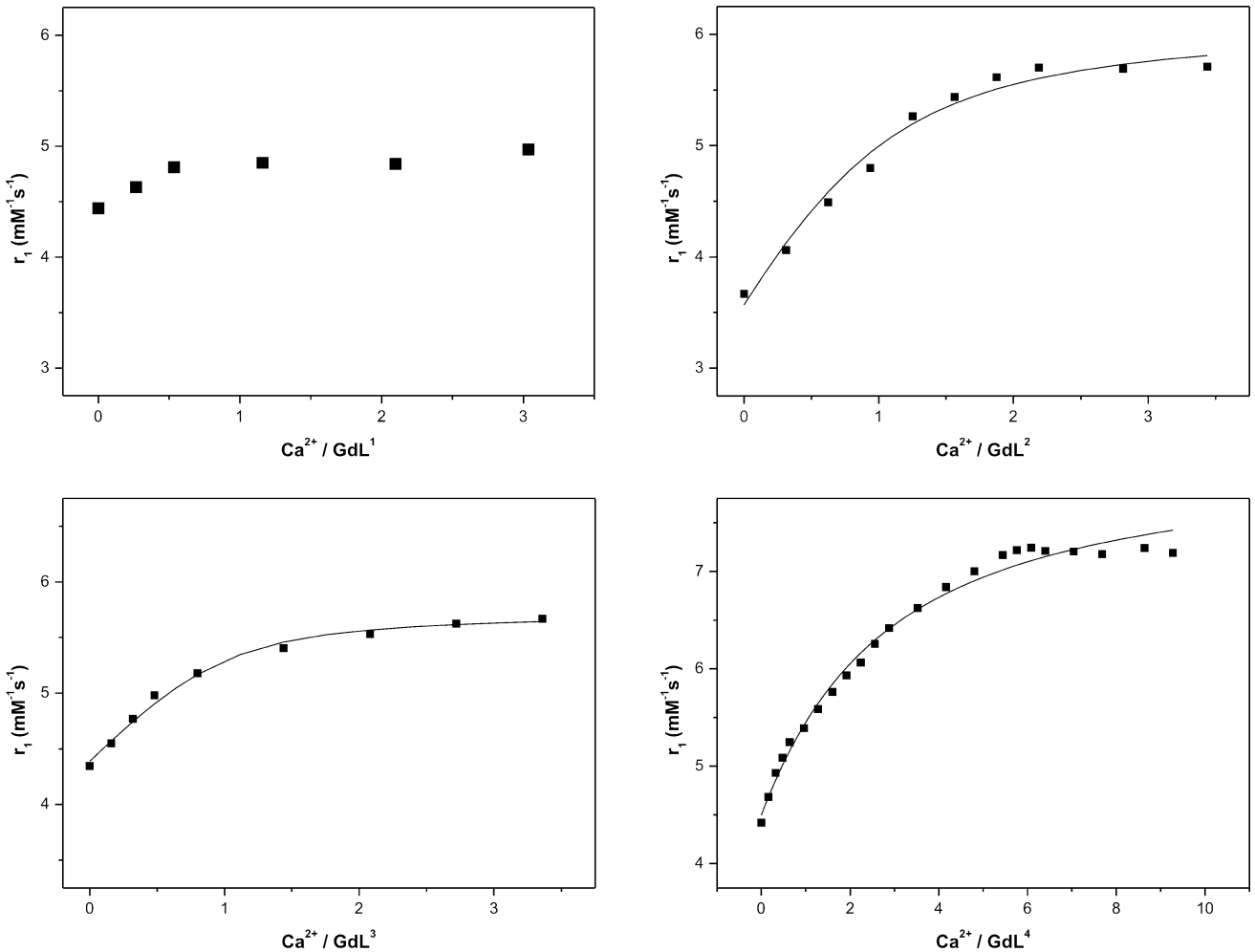


Fig. 1 Relaxometric titration of **GdL$^{1-4}$** with Ca^{2+} at 7 T, 25 °C, pH 7.3 (HEPES). The curves correspond to the fit as explained in the text.

of **GdL^2**, which is probably caused by the absence of additional coordination sites in the side chain of **GdL^2**. Its ethylenediamine core appended with three carboxylates can interact with several metal ions, however it does not show selectivity over a particular ion.

A similar behavior was demonstrated in the comparative analysis of the r_1 responses of **GdL^3**. Namely, the addition of Mg^{2+} and Cu^{2+} increases the r_1 for 20% and 24%, respectively. The level of these changes is of a similar order as observed for Ca^{2+} (30%). A slightly higher r_1 enhancement upon the addition of Ca^{2+} is presumably due to the presence of the two ether oxygens and the size of the binding site, which can coordinate more effectively to Ca^{2+} over the other two cations. However, the high flexibility of the side chain and the longer distance of the iminodiacetate moiety from the reporting moiety may also contribute to the weaker r_1 response.

GdL^4 exhibits the highest and the most selective response towards Ca^{2+} in the presence of investigated competing cations. The total r_1 enhancement is 63%, as in the initial relaxometric titration experiment. The addition of Mg^{2+} (10 equiv.) to the buffered solution of **GdL^4** leads to a slight increase in r_1 (17%). The presence of the other metal ions in the system has no noticeable effect on the r_1 changes. Interestingly, the structure of the side chain in this complex is very similar to that of **GdL^3**, except that aliphatic dimethylene group is replaced with the two aromatic carbons of the catechol group. This obviously contributes to the increase of the selectivity of **GdL^4** *versus* **GdL^3** towards Ca^{2+}, as well as in higher r_1 changes upon the addition of this ion.

Luminescence emission lifetimes

Luminescence lifetime measurements for the complexes **EuL^1**–**EuL^4** were recorded in H_2O and D_2O in the absence and presence of Ca^{2+}. The number of the inner sphere water molecules (q) was calculated from the empirical equation expressed in the eqn (2), where $A = 1.2$ and $B = 0.25$ for Eu^{3+}.[22]

$$q = A \times (\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - B) \quad (2)$$

The data obtained provided a useful insight into the europium coordination environment and the number of coordinated water molecules (Table 1). **EuL^1** exists in solution as a mono-aqua complex which is rather to be expected, considering the number of donor atoms available. This hydration state does not change also after addition of 3 equivalents of Ca^{2+} to the solution of **EuL^1** in buffer, which supports the results from the relaxometric titrations where minor r_1 changes were observed (see above). The hydration

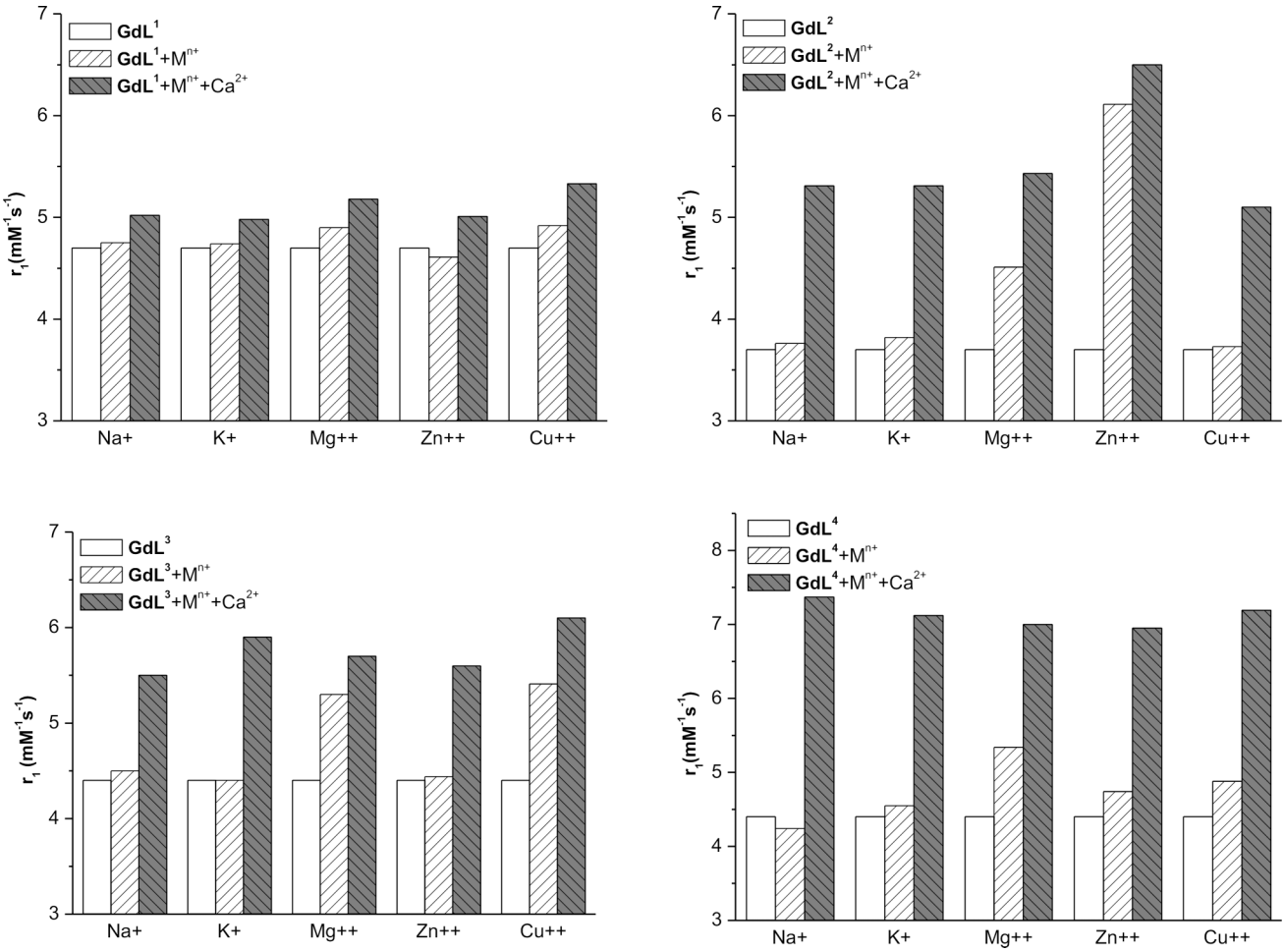


Fig. 2 Comparison of the r_1 response of **GdL$^{1-4}$** in presence of Na^+, K^+, Mg^{2+}, Zn^{2+} and Cu^{2+}, before and after the addition of Ca^{2+} (7 T, 25 °C, pH 7.4, HEPES buffer).

Table 1 Emission lifetimes and estimated q values of Eu^{3+} complexes in the presence and absence of Ca^{2+} (25 °C, pH 7.4, HEPES buffer)

Compound	τ (ms) H_2O	τ (ms) D_2O	q	Compound	τ (ms) H_2O	τ (ms) D_2O	q
EuL^1	0.54	1.27	1.0	**EuL^1+** 3 equiv. Ca^{2+}	0.55	1.29	0.9
EuL^2	0.70	0.99	0.2	**EuL^2+** 3 equiv. Ca^{2+}	0.57	1.16	0.8
EuL^3	0.51	0.65	0.2	**EuL^3+** 3 equiv. Ca^{2+}	0.47	0.64	0.4
EuL^4	0.80	1.17	0.2	**EuL^4+** 6 equiv. Ca^{2+}	0.38	1.29	1.9

state of other three complexes **EuL^2**–**EuL^4** is close to zero in the absence of Ca^{2+}, and it increases upon the addition of Ca^{2+}. The amplitude of changes in q correspond to those observed in r_1. The smallest change in q (for **EuL^3**) is associated with the smallest r_1 enhancement of **GdL^3** and the highest changes in q for **EuL^4** are followed with the highest r_1 changes in **GdL^4**. However a more detailed physicochemical study would be required to fully explain the r_1 behaviour of the bishydrated **EuL^4** + Ca^{2+} species.

Conclusions

Four novel DO3A-based macrocyclic ligands appended with different side chains and their Gd^{3+}/Eu^{3+} complexes were synthesized *via* four convenient routes. The systems display varying interactions with Ca^{2+}, and their response to several endogenous metal ions was also investigated. The r_1 of **GdL^2**–**GdL^4** increases after addition of Ca^{2+}, whereas no analogous changes are observed for the amide containing **GdL^1**. The levels of the r_1 response, as well as the selectivity of **GdL^2**–**GdL^4** towards Ca^{2+} and/or Mg^{2+}, Zn^{2+}, Cu^{2+}, depend on the structure of the side chain, the number of coordination sites and its flexibility. The advantage gained by the easy synthesis of **L^1**–**L^4** by selecting simple precursors for chelators is apparently outweighed by the loss of binding sites that could optimise both the r_1 response and the selectivity towards the investigated metal ions. **EuL^2**–**EuL^4** have a low hydration number as determined by luminescence lifetime experiments, similar to that observed previously for monomacrocylic SCA with similar structures.[9,11,12] The hydration number of these complexes increases upon the addition of Ca^{2+} and Mg^{2+}, Zn^{2+} or Cu^{2+}, which corresponds with the r_1 changes observed for the analogous

Gd^{3+} complexes. The exception to this is **EuL^1**, where q remains at a constant value of one throughout the experiments, which is explained by the presence of a strong coordination between the amide and paramagnetic ion in this complex. This study has revealed much useful information for the future design of metal sensitive SCA and potential improvements to their structure.

Experimental section

General

All chemicals were purchased from commercial sources and were used without further purification. All dried solvents were stored under argon. The lanthanide metal ion solutions with known concentrations were prepared by dissolving an accurately weighed amount of the chloride salt in the appropriate volume of doubly distilled water, and standardized with the complexometric titration with Na_2EDTA. ^{1}H NMR, and $^{13}C\{^1H\}$ NMR spectra were recorded on Bruker DRX300 spectrometer at room temperature. Mass spectra (ESI-LRMS in positive and negative ion mode) were performed on an ion trap SL 1100 system (Agilent, Germany). FT-ICR-MS were performed on a Bruker FT-ICR Apex II spectrometer. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed at room temperature on a Varian PrepStar Instrument, Australia, equipped with PrepStar SD-1 pump heads. UV absorbance was measured using a ProStar 335 photodiode array detector at 214 and 254 nm. Analytical RP-HPLC was performed in a stainless steel Chromsep (length 250 mm, internal diameter 41.4 mm, outside diameter 2 inch and particle size 8 μm) C_{18} column (Varian, Advanced Chromatographic Solutions). Luminescence lifetime measurements were performed on a QuantaMaster™ 3 PH fluorescence spectrometer from Photon Technology International, Inc., USA. Column chromatography was performed using silica gel 60 (70–230 mesh ASTM) from Fluka, Germany.

Tri-*tert*-butyl-2,2′,2″-(10-(2-((2-*tert*-butoxy-2-oxoethyl)(2-(*tert*-butoxycarbonylamino)ethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (2). Tris-*t*Bu-DO3A (0.514 g, 1.0 mmol) and K_2CO_3 (0.138 g, 1.0 mmol) were dissolved in acetonitrile (30 ml); to this a solution of **1** (0.473 g, 1.2 mmol) in acetonitrile (10 ml) was added. After the reaction mixture was stirred for 12 h at 70 °C, the solution was allowed to cool to r.t., the inorganic salts were removed by filtration and the solvent was concentrated under reduced pressure to give a crude product as a yellow oil. This was purified by column chromatography (silica gel, 5% of MeOH in CH_2Cl_2) to give 0.605 g (73%) of **2** as yellow viscous oil. ^{1}H NMR ($CDCl_3$): δ 1.22–1.29 (br, 45H, CH_3), 1.85 (bs, 6H, CH_2), 3.03–3.39 (br, 16H, CH_2), 3.62–3.94 (bs, 6H, CH_2), 5.66 (bs, 2H, CH_2). ^{13}C NMR ($CDCl_3$): δ 25.8, 25.9, 26.4, 26.5 (CH_3), 36.7, 46.4, 47.1, 47.7, 48.0, 50.0, 50.3, 53.4, 54.3, 54.7 (CH_2), 78.6, 80.1, 81.3, 81.5 ($C(CH_3)_3$), 155.6, 155.7 (CONH), 168.2, 171.7, 171.6 (*C*OOtBu). ESI-MS: for $C_{41}H_{76}N_6O_{11}$ calcd. 829.6 $[M + H]^+$, found: 829.6.

Tri-*tert*-butyl 2,2′,2″-(10-(2-((2-(bis(2-*tert*-butoxy-2-oxoethyl)-amino)ethyl)(2-*tert*-butoxy-2-oxoethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (3). A methanolic solution of **2** in 4 M HCl was stirred at r.t. for 20 min. The methanol was evaporated under reduced pressure and the residue was dissolved in 30 ml of acetonitrile. K_2CO_3 (0.276 g, 2.0 mmol) was added to the solution and the reaction mixture was stirred at r.t. for 30 min. *tert*-Butyl bromoacetic acid (0.347 g, 1.75 mmol) was added dropwise into the mixture which was stirred at 70 °C for 12 h. The reaction mixture was allowed to cool to r.t., the inorganic salts were removed by filtration and the solvent concentrated under reduced pressure to give the crude product as an brown oil which was purified by column chromatography (5% of MeOH in CH_2Cl_2) to give the final product **3** as a yellow oil (0.49 g, 70%). ^{1}H NMR ($CDCl_3$): δ 1.09–1.15 (br, 54H, CH_3), 1.14–3.92 (br, 24H, CH_2), 3.38 (br, 6H, CH_2), 3.92 (br, 4H, CH_2). ^{13}C NMR ($CDCl_3$): δ 27.5, 27.6, 27.7, 27.8 (CH_3), 49.2, 50.0, 50.7, 52.7, 53.6, 55.3, 56.2, 57.8, 60.8, 65.4, 65.7 (CH_2), 81.9, 82.1, 82.2, 82.6 ($C(CH_3)_3$), 154.9 (CONH), 172.1, 172.4, 172.8, 173.3 (*C*OOtBu). ESI-MS: for $C_{48}H_{88}N_6O_{13}$ calcd. 957.6 $[M + H]^+$, found: 957.6.

2,2′,2″-(10-(2-((2-(Bis(carboxymethyl)amino)ethyl)(carboxymethyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (L^1). **3** (2.01 g, 2.1 mmol) was dissolved in dichloromethane (20 ml) and trifluoroacetic acid (20 ml) was added slowly. After the mixture had been stirred at r.t. for 24 h, the solvent was removed under reduced pressure. Dichloromethane (40 ml) was added and evaporated twice in order to remove the excess of trifluoroacetic acid. The same procedure was repeated twice with methanol. The viscous residue was dissolved in a minimum amount of methanol and cold diethyl ether was added dropwise. The resulting precipitate was isolated by filtration and resuspended in water (3 ml). A large excess of acetone (100 ml) was added and the cloudy solutions were stored at –20 °C for 16 h. The colorless crystalline powder was isolated by filtration, washed with acetone and dried under reduced pressure. (0.93 g, 71%). ^{1}H NMR (D_2O): δ 1.22 (bs, 2H, CH_2NCO), 1.49–1.57 (br, 6H, CH_2), 2.78–3.05 (br, 12H, CH_2COOH), 3.18–3.43 (br, 12H, CH2), 3.75 (s, 2H, CH_2). ^{13}C NMR (D_2O): δ 22.7, 22.9, 28.4, 39.2, 48.3, 48.5, 51.4, 51.6, 53.2, 54.1, 56.0 (CH_2), 162.8, 163.0, 169.8, 174.4 (COOH). FT-ICR-MS: for $C_{24}H_{40}N_6O_{13}$ calcd. 619.2581 $[M - H]^+$, found: 619.2656.

Tri-*tert*-butyl 10-(2-(2-(*tert*-butoxycarbonylamino)ethylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (5). A solution of **4** (2.53 g, 9.0 mmol) in acetonitrile (30 ml) was added to a solution of tris-Boc-cyclen (3.78 g, 8.0 mmol) and K_2CO_3 (1.4 g, 10 mmol) in acetonitrile (80 ml). After the reaction mixture was stirred for 12 h at 70 °C, the solution was allowed to cool to r.t., the inorganic salts were removed by filtration and the solvent concentrated under reduced pressure to give a yellow oil. The oil was purified by column chromatography (15% of ethyl acetate in DCM) to give 4.20 g (78%) of **5** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.40–1.42 (br, 36H, CH_3), 1.97 (bs, 4H, CH_2), 2.79 (s, 2H, CH_2), 3.21–3.42 (br, 12H, CH_2), 3.58 (s, 4H, CH_2). ^{13}C NMR ($CDCl_3$): δ 28.2, 28.3, 28.5 (CH_3), 30.7, 32.6, 38.8, 39.7, 45.8, 49.3, 50.8 (CH_2), 79.0, 79.1, 79.2 ($C(CH_3)_3$), 155.2, 155.4, 155.8 (*C*ONH). ESI-MS: for $C_{32}H_{60}N_6O_9$ calcd. 672.4 $[M + H]^+$, found: 672.3.

***N*1-(2-(1,4,7,10-Tetraazacyclododecan-1-yl)ethyl)ethane-1,2-diamine (6).** 4 N HCl in methanol (50 ml) was added to the compound **5** (4.20 g, 6.2 mmol) and the solution was stirred for 1 h at r.t. The solvent was evaporated and the resulting white solid was resuspended in dry THF (20 ml) and added slowly to a

stirring suspension of $LiAlH_4$ (0.23 g, 6.2 mmol) in dry THF (50 ml) under N_2 at 0 °C. Following the complete addition, the reaction mixture was heated to reflux temperature for 6 h. The mixture was then cooled in an ice-bath and the reaction product and excess of hydride precipitated from solution. The hydride was quenched by the dropwise addition of water followed by 15% sodium hydroxide. After vigorous stirring for another 20 min, further impurities were removed by filtration. The filtrate was concentrated and dried overnight under reduced pressure, and the yellow oil (**6**, 0.9 g, 70%) was used in the next step without purification. ^{1}H NMR (D_2O): δ 2.67–2.96 (br, 10H, CH_2), 3.19–3.40 (br, 10H, CH_2), 3.7 (br, 4H, CH_2). ^{13}C NMR (D_2O): δ 37.4, 48.7, 49.8, 50.6, 51.6, 53.8, 56.5 (CH_2). ESI-MS: for $C_{12}H_{30}N_6$ calcd. 259.2 $[M + H]^+$, found: 259.3.

Tri-*tert*-butyl 2,2′,2″-(10-(2-((2-(bis(2-*tert*-butoxy-2-oxoethyl)-amino)ethyl)(2-*tert*-butoxy-2-oxoethyl)amino)ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (7). **6** (0.9 g, 3.6 mmol) and K_2CO_3 (2.5 g, 18 mmol) were dissolved in acetonitrile (70 ml); to this a solution of 4.9 g (25 mmol) *tert*-butyl bromoacetate in acetonitrile (30 ml) was added. After the reaction mixture was stirred for 12 h at 70 °C, the solution was allowed to cool to r.t., the inorganic salts were removed by filtration and the solvent was concentrated under reduced pressure to give the crude product as a brown oil. The oil was purified by column chromatography (5% of MeOH in CH_2Cl_2) to give 2.5 g (76%) of **7** as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.31–1.34 (2 × bs, 63H, CH_3), 2.12–3.32 (br, 36H, CH_2). ^{13}C NMR ($CDCl_3$): δ 27.7, 27.8, 27.9, 28.0 (CH_3), 50.0, 50.1, 50.7, 51.8, 51.9, 52.3, 53.5, 55.5, 55.7, 55.9, 56.1, 56.3, (CH_2), 80.7, 81.8, 82.3, 82.6 ($C(CH_3)_3$), 170.3, 170.4, 172.5, 172.8 (*C*OOtBu). ESI-MS: for $C_{48}H_{90}N_6O_{12}$ calcd. 943.7 $[M + H]^+$, found: 943.8.

2,2′,2″-(10-(2-((2-(Bis(carboxymethyl)amino)ethyl) (carboxymethyl)amino)ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)-triacetic acid (L^2). **7** (2.1 g, 2.2 mmol) was dissolved in formic acid (20 ml) and the mixture was stirred at 60 °C for 24 h. The solvent was removed under reduced pressure and the viscous residue was dissolved in a minimum amount of methanol and added dropwise to cold diethyl ether. The resulting precipitate was isolated by filtration and resuspended in 3 ml of water. A large excess of acetone (100 ml) was added and the cloudy solution was stored at –20 °C for 16 h. A colorless crystalline powder was isolated by filtration, washed with acetone and dried under reduced pressure to give $\mathbf{L^2}$ (0.225 g, 71%). ^{1}H NMR (D_2O): δ 2.99–4.07 (br, 36H). ^{13}C NMR (D_2O): δ 45.7, 46.5, 47.5, 49.1, 49.3, 49.6, 50.6, 52.9, 54.0, 56.5, 56.6, 57.1 (CH_2), 169.9, 173.9, 174.1, 174.2 (COOH) ESI-MS: for $C_{24}H_{42}N_6O_{12}$ calcd. 605.2788 $[M + H]^-$, found: 605.2790.

Tri-*tert*-butyl 2,2′,2″-(10-(2-(2-(2-(1,3-dioxoisoindolin-2-yl)-ethoxy)ethoxy)ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)-triacetate (9). Tris-*t*Bu-DO3A (1.00 g, 1.94 mmol) and K_2CO_3 (0.40 g, 2.9 mmol) were dissolved in acetonitrile (30 ml); to this a solution of **8** (0.79 g, 2.32 mmol) in acetonitrile (20 ml) was added. After the reaction mixture had been stirred for 16 h at 70 °C, the solution was allowed to cool to r.t., the inorganic salts were removed by filtration and the solvent was concentrated under reduced pressure to give a brown viscous oil. The compound was purified by column chromatography (silica gel, 5% MeOH in CH_2Cl_2) to yield 1.05 g (70%) of **9** as yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.38 (s, 9H, CH_3), 1.40 (s, 18H, CH_3), 2.24–3.80 (br, 34H, CH_2), 7.72–7.78 (br, 4H, C_6H_4). ^{13}C NMR ($CDCl_3$): δ 27.8, 27.9 (CH_3), 37.1, 42.8, 49.4, 50.2, 52.0, 53.5, 55.5, 56.2, 67.0, 67.4, 69.5, 69.9 (CH_2), 82.0, 82.6 ($C(CH_3)_3$), 123.1, 131.7, 134.3 (C_6H_4), 168.0 (NCO), 172.4, 172.8 (*C*OOtBu). FT-ICR-MS: for $C_{40}H_{65}N_5O_{10}$ calcd. 776.4804 $[M + H]^+$, found: 776.4799.

Tri-*tert*-butyl 2,2′,2″-(10-(9-(2-*tert*-butoxy-2-oxoethyl)-13,13-dimethyl-11-oxo-3,6,12-trioxa-9-azatetradecyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (10). Compound **9** (1.00 g, 1.29 mmol) was dissolved in 10 ml of a mixture of ethylenediamine and isopropanol (1 : 1). The solution was heated to reflux temperature for 16 h, and then was allowed to cool to r.t. The solvent was removed under reduced pressure and the remainingethylenediaminee was removed using the bulb-to-bulb distillation apparatus. The resulting viscous oil was dissolved in the 30 ml of acetonitrile. Following the addition of K_2CO_3 (0.43 g, 3.00 mmol) the solution was stirred for 30 min at r.t. *tert*-Butyl bromoacetate (0.61 g, 3.01 mmol) in acetonitrile (50 ml) was added dropwise and the mixture was stirred for 18 h at 70 °C. The inorganic salts were removed by filtration and the solvent was evaporated under reduced pressure. The light brown viscous oil was purified by column chromatography (silica gel, 5% MeOH in CH_2Cl_2) to yield 0.85 g (75%) of **10** as brown viscous oil. ^{1}H NMR ($CDCl_3$): δ 1.38 (br, 27H, CH_3), 1.43 (s, 18H, CH_3), 2.64 (s, 4H, CH_2), 3.01–4.39 (br, 34H, CH_2). ^{13}C NMR ($CDCl_3$): δ 27.9, 28.0, 28.1 (CH_3), 35.0, 37.8, 39.1, 45.3, 45.4, 49.2, 49.5, 50.7, 51.5, 53.2, 56.0, 56.8, 61.8 (CH_2), 81.8, 82.2, 83.8 ($C(CH_3)_3$), 163.8, 166.1, 171.8 (*C*OOtBu). FT-ICR-MS: for $C_{44}H_{83}N_5NaO_{12}$ calcd. 896.5930 $[M + Na]^+$, found: 896.5930.

2,2′,2″-(10-(2-(2-(2-(Bis(carboxymethyl)amino)ethoxy)ethoxy)-ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (L^3). Compound **10** (0.85 g, 0.98 mmol) was dissolved in CH_2Cl_2 (5 ml) and TFA (3 ml) was added slowly. After the mixture was stirred at rt for 24 h, the solvent was removed under reduced pressure. CH_2Cl_2 (40 ml) was added and evaporated twice to remove the excess of trifluoroacetic acid and volatiles. The same procedure was repeated twice with methanol. The viscous residue was dissolved in a minimum amount of methanol and cold diethyl ether was added dropwise. The formed precipitates were filtered, washed with cold ether and dried *in vacuo*. A light brown hygroscopic powder was obtained ($\mathbf{L^3}$, 0.4 g, 69%).

^{1}H NMR (D_2O): δ 3.03 (br, 4H, CH_2), 3.21 (s, 6H, CH_2), 3.36–3.56 (br, 18H CH_2), 3.78 (bs, 2H CH_2), 3.91–3.94 (br, 8H CH_2). ^{13}C NMR (D_2O): δ 37.1, 48.3, 48.8, 50.8, 51.3, 52.2, 53.0, 55.2, 56.5, 57.2, 64.6, 69.6, 70.0 (CH_2), 164.9, 168.5, 169.2 (*C*OOH). FT-ICR-MS: for $C_{28}H_{43}N_5O_{12}$ calcd. 594.2978 $[M + H]^+$, found: 594.2980.

Benzyl 2-(2-(2-hydroxyethoxy)phenoxy)ethylcarbamate (11). A solution of *N*-benzyloxycarbonyl-2-bromoethylamine (4.0 g, 16 mmol) in DMF (20 ml) was added to a mixture of 2-(2-hydroxyethoxy)phenol (2.0 g, 13 mmol) and K_2CO_3 (2.2 g, 16 mmol) in DMF (30 ml). The reaction mixture was stirred for 16 h at reflux temperature. The solution was allowed to cool to r.t., the inorganic salts were removed by filtration and the solvent was concentrated under reduced pressure to give brown viscous oil. The compound was purified by column chromatography (silica gel, 20% EtOAc in CH_2Cl_2) to yield 2.66 g (63%) of **11** as a brown

oil. ^{1}H NMR ($CDCl_3$): δ 3.46 (bs, 2H, CH_2), 3.79 (s, 2H, CH_2), 3.95 (s, 4H, CH_2), 5.00 (s, 2H, CH_2), 6.00 (s, 1H, NH), 6.82 (s, 4H, C_6H_4), 7.23 (s, 5H, C_6H_5), 7.88 (s, 1H, C_6H_5). ^{13}C NMR ($CDCl_3$): δ 21.0, 40.7, 61.0, 66.7, 68.9, 71.2, (CH_2), 115.1, 122.1, 128.1, 128.5, 136.7, 148.7, 148.9 (C_6H_4 and C_6H_5), 158.6 (NHCOO). FT-ICR-MS: for $C_{18}H_{21}NNaO_5$ calcd. 354.1312 $[M + Na]^+$, found: 354.1309.

Tri-*tert*-butyl 2,2′,2″-(10-(2-(2-(2-(benzyloxycarbonylamino)-ethoxy)phenoxy)ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)-triacetate (12). Compound **11** (1.0 g, 3.0 mmol) was dissolved in dry cyclohexane (10 ml) under a nitrogen atmosphere. Dry DMF (0.5 ml) was added to the solution followed by a dropwise addition of the thionyl bromide (1.2 g, 6.0 mmol). The reaction mixture was stirred at r.t. for 3 h, after which dichloromethane (30 ml) was added to the mixture and the excess thionyl bromide was quenched by the addition of a saturated solution of $NaHCO_3$. The organic phase was washed with brine (2 × 50 ml), water (1 × 50 ml), dried over sodium sulphate and concentrated *in vacuo*. The resulting brown oil was dissolved in acetonitrile (20 ml) and added dropwise to the mixture of tris-*t*Bu-DO3A (1.03 g, 2.0 mmol) and K_2CO_3 (0.40 g, 2.9 mmol) in acetonitrile (30 ml). After the reaction mixture had been stirred for 16 h at 70 °C, the solution was allowed to cool to r.t., the inorganic salts were removed by filtration and the solvent was concentrated under reduced pressure to give a brown viscous oil. The compound was purified by column chromatography (silica gel, 5% MeOH in CH_2Cl_2) to yield 1.05 g (70%) of **12** as yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.31 (br, 27H, CH_3), 2.09–3.61 (br, 26H, CH_2), 4.08 (s, 2H, CH_2), 5.03 (s, 2H, CH_2), 5.25 (s, 2H, CH_2), 6.20 (bs, 1H, C_6H_4), 6.71 (bs, 1H, C_6H_4), 6.84 (br, 2H, C_6H_4), 7.27 (br, 5H, C_6H_5). ^{13}C NMR ($CDCl_3$): δ 27.6, 27.7 (CH_3), 40.3, 49.7, 50.2, 51.7, 51.7, 53.6, 55.3, 56.0, 66.1, 67.1, 68.1 (CH_2), 81.9, 82.1 ($C(CH_3)_3$), 113.2, 118.6, 121.1, 123.4, 127.7, 128.3, 136.7, 148.1, 149.8, 156.6, (C_6H_5 and C_6H_4), 172.3, 173.0, (*C*OOtBu). FT-ICR-MS: for $C_{44}H_{69}N_5O_{10}$ calcd. 828.5234 $[M + H]^+$, found: 828.5234.

Tri-*tert*-butyl 2,2′,2″-(10-(2-(2-(2-(bis(2-*tert*-butoxy-2-oxo-ethyl)amino)ethoxy)phenoxy)ethyl) - 1,4,7,10 - tetraaza - cyclododecane-1,4,7-triyl)triacetate (13). Compound **12** (1.00 g, 1.21 mmol) was dissolved in EtOH (30 ml). Pd/C was added and mixture was placed into the Parr hydrogenator under 3 bar of H_2 pressure for 12 h. The reaction mixture was filtered through celite, concentrated under reduced pressure and dissolved in acetonitrile (30 ml). K_2CO_3 (0.37 g, 2.6 mmol) was added and the mixture was stirred for 20 min at r.t. followed by the dropwise addition of the solution of *tert*-butyl bromoacetate (0.52 g, 2.6 mmol) in acetonitrile (20 ml). The reaction mixture was stirred for 16 h at reflux temperature. The solution was allowed to cool to r.t., filtered and concentrated under reduced pressure to give a brown viscous oil. The compound was purified by column chromatography (silica gel, 5% MeOH in CH_2Cl_2) to yield 0.81 g (73%) of **13** as light brown oil. ^{1}H NMR ($CDCl_3$): δ 1.02 (s, 18H, CH_3), 1.10 (s, 18H, CH_3), 1.18 (s, 9H, CH_3), 1.96–2.91 (br, 22H, CH_2), 3.09 (bs, 12H, CH_2), 6.51 (s, 1H, C_6H_4), 6.63 (br, 3H, C_6H_4). ^{13}C NMR ($CDCl_3$): δ 27.5, 27.6, 27.7 (CH_3), 49.6, 49.8, 50.1, 50.3, 51.7, 55.2, 55.4, 55.6, 55.9, 59.2, 67.9 (CH_2), 81.8, 81.9, 82.1 ($C(CH_3)_3$), 117.2, 121.1, 122.9, 128.5, 129.8, 148.0 (C_6H_4), 172.2, 173.0, 173.1 (*C*OOtBu). FT-ICR-MS: for $C_{44}H_{83}N_5O_{12}$ calcd. 922.4749 $[M + H]^+$, found: 922.4749.

2,2′,2″-(10-(2-(2-(2-(Bis(carboxymethyl)amino)ethoxy)-phenoxy)ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (L^4). Compound **13** (0.81 g, 0.88 mmol) was dissolved in formic acid (20 ml) and the mixture was stirred at 60 °C for 24 h. The solvent was removed under reduced pressure and the viscous residue was dissolved in a minimum amount of methanol and added dropwise to cold diethyl ether. The resulting precipitate was isolated by filtration and resuspended in 3 ml of water. A large excess of acetone (100 ml) was added and the cloudy solution was stored at –20 °C for 16 h. The white crystalline powder was isolated by filtration, washed with acetone and dried under reduced pressure resulting in 0.39 g (70%) of the final compound $\mathbf{L^4}$. ^{1}H NMR (D_2O): δ 3.01–3.64 (br, 26H, CH_2), 3.90 (bs, 4H, CH_2), 4.29 (br, 4H, CH_2), 6.91 (br, 4H, C_6H_4). ^{13}C NMR (D_2O): δ 47.7, 48.1, 49.1, 49.5, 51.4, 51.6, 52.9, 53.3, 53.8, 55.7, 61.7 (CH_2), 112.6, 112.7, 121.1, 121.5, 145.4, 145.9 (C_6H_4), 164.7, 168.3, 171.3 (*C*OOH). FT-ICR-MS: for $C_{28}H_{43}N_5O_{12}$ calcd. 640.2782 $[M - H]^+$, found: 640.2777.

General procedure for preparation of GdL^{1-4} and EuL^{1-4}

$\mathbf{L^{1-4}}$ (~50–100 μmol) was dissolved in water (5 ml) and a stock solution with known concentration of $GdCl_3$ or $EuCl_3$ (1 equiv.) was added. The solutions were heated to 60 °C for 24 h and the pH was maintained at ~7 using NaOH. The complexes were treated with Chelex 100 to remove the excess lanthanide ions. The resulting solutions were filtered and lyophilized to give $\mathbf{GdL^{1-4}}$ or $\mathbf{EuL^{1-4}}$.

GdL^1 ESI-MS: for $C_{24}H_{35}GdN_6NaO_{13}^-$ calcd. 796.1 [M + Na – 2H]$^-$, found: 796.2. **EuL^1** ESI-MS: for $C_{24}H_{36}EuN_6O_{13}^-$ calcd. 769.2 [M – H]$^-$, found: 768.2. **GdL^2** ESI-MS: for $C_{24}H_{37}GdN_6NaO_{12}^-$ calcd. 782.2 [M + Na – 2H]$^-$, found: 782.2. **EuL^2** ESI-MS: for $C_{24}H_{37}EuN_6NaO_{12}^-$ calcd. 777.2 [M + Na – 2H]$^-$, found: 777.1. **GdL^3** ESI-MS: for $C_{24}H_{38}GdN_5NaO_{12}^-$ calcd. 769.2 [M + Na – 2H]$^-$, found: 769.2. **EuL^3** ESI-MS: for $C_{24}H_{38}EuN_5NaO_{12}^-$ calcd. 764.2 [M + Na – 2H]$^-$, found: 764.2. **GdL^4** ESI-MS: for $C_{28}H_{38}GdN_5NaO_{12}^-$ calcd. 817.2 [M + Na – 2H]$^-$, found: 817.2. **EuL^4** ESI-MS: for $C_{28}H_{38}EuN_5NaO_{12}^-$ calcd. 812.2 [M + Na – 2H]$^-$, found: 812.0.

Relaxometric titrations

The titrations were performed at 7 T, 25 °C and pH 7.3–7.4 (maintained by HEPES buffer). A solution of metal ion salt ($CaCl_2$, $MgCl_2$, $ZnCl_2$, NaCl, KCl, $CuSO_4$) of known concentration was added in stepwise portions to the complex solution and the longitudinal proton relaxation time (T_1) was measured after each addition of the analyte. The relaxivity (r_1) was calculated from eqn (1), using the actual Gd^{3+} concentration at each point of the titration. The initial Gd^{3+} concentrations were determined by measuring the bulk magnetic susceptibility shifts.[23] The titration curves were fitted according to the modified equation previously reported to obtain the apparent association constants.[24]

Luminescence lifetime experiments

The luminescence decay experiments were performed in H_2O and D_2O (25 °C, pH 7.3, HEPES) on $\mathbf{EuL^1}$–$\mathbf{EuL^4}$ at 5 mM

concentrations. The Eu^{3+} ion was directly excited at 395 nm and emission intensity at 615 nm was recorded with 10 μs resolution. Excitation and emission slits were set to 15 and 5 nm bandpass respectively. Datasets are an average of 25 scans and each reported value is the mean of three independent measurements. Obtained curves are fitted to the first order exponential decay with $r^2 = 0.99$. q values are calculated using eqn (2).

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