Synthesis and characterisation of bismacroyclic DO3A-amide derivatives – an approach towards metal-responsive PARACEST agents†

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Three new bismacroyclic Ln3+ chelates consisting of triamide derivatives of cyclen with glycine, methyl and tert-butyl substituents (L1−3, respectively) linked to an acyclic EGTA-derived calcium chelator were synthesised as potential MRI contrast agents (EGTA – ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid). Eu3+ and Yb3+ complexes of L1−3 were investigated as chemical exchange saturation transfer (CEST) agents. Moderate to minor CEST effects were observed for Eu2L1, Eu2L2 and Yb2L2 complexes in the absence of Ca2+, with negligible changes upon addition of this metal ion. Luminescence steady-state emission and lifetime experiments did not reveal any changes in the coordination environment of the complexes, while the number of inner-sphere water molecules remained constant in the absence and presence of Ca2+. The protonation constants of Eu2L1 and Eu2L2 and stability constants of their complexes with Ca2+, Mg2+ and Zn2+ were determined by means of potentiometric titrations. The results show that the charge of the complex dramatically affects the protonation constants of the EGTA-binding unit. The stability constants of the complexes formed with Ca2+, Mg2+ and Zn2+ are several orders of magnitude lower than those of EGTA. These findings indicate that the nature of Ln3+ chelates and their charge are the main reasons for the observed results and weaker response of these EGTA-derived triamide derivatives compared to their tricarboxylate analogues.

Introduction

The continuous development of contrast agents (CAs) for magnetic resonance imaging (MRI) has provided a wide range of structurally different compounds with a range of diagnostic and therapeutic applications.1,2 The first generation and the most widely used MRI contrast agents are based on paramagnetic Gd3+ complexes or superparamagnetic iron–oxide nanoparticles (T1- and T2-shortening agents, respectively). Although these CAs remain extensively investigated in basic research and in clinical medicine, they exhibit certain limitations related to their lack of tissue specificity and response to the chemical environment. Therefore, alternative approaches for producing image contrasts that provide additional information are greatly appreciated, leading to the development of several methodologies based on novel types of CAs.3 Among these, the mechanism for altering MR contrast based on chemical exchange saturation transfer (CEST) has been recently established.4 This technique has been known and used in nuclear magnetic resonance (NMR) for more than four decades, however only lately it has attracted greater attention due to its capability to generate an MRI contrast on its own, and also due to its high sensitivity towards changes in the microenvironment.5 CEST imaging requires sufficiently slow exchange on the magnetic resonance time scale to allow selective irradiation of the protons of interest. The rate of exchange (kex) that occurs between the two magnetically distinct environments must not be greater than the difference in frequency between them (∆ω0), while several other physicochemical parameters can also affect CEST MR contrast mechanisms, including the relaxation rates of the two pools involved in chemical exchange, temperature and concentration.6 Furthermore, a methodology that exploits particular classes of paramagnetic...
lanthanide complexes for introducing tissue contrast via a CEST mechanism has also been developed. These complexes are specifically designed to shift exchangeable protons (–NH, –OH, –SH or bound water) further away from the bulk water allowing their distinct saturation, consequently reducing the intensity of the bulk water MR signal and hence producing the change in MRI contrast.\(^7\)

The dependence of CEST contrast on diverse factors, including those involving paramagnetic (PARACEST) agents, can be used for the detection of specific biological processes in tissues by means of MRI. Consequently, various molecular imaging probes responsive to particular molecular events – the so called bioreponsive or “smart contrast agents” (SCAs) – have been designed.\(^2\) In most of the cases, MRI signal changes are triggered by the variation in hydration number or rotation dynamics for the \(T_1\)– and \(T_2\)-based CA. On the other hand, signal differences produced by CEST agents are caused by the exchange rate and the chemical shift of the exchangeable proton pool, making CEST agents extremely sensitive to environmental changes, and leading to the fast development of responsive CEST agents. The most widely investigated probes of this class show response to pH and temperature, which allow a direct read-out of these relevant physiological parameters in the disease state.\(^8\) However, systems that provide responses to metabolites, biologically relevant ions, enzyme or redox activity have also been reported.\(^6\)

The usage of SCAs in MRI for observing specific biological processes is an extremely promising and potentially very beneficial approach to study various functional processes at the molecular and cellular level. For example, successful monitoring of \(\text{Ca}^{2+}\) would be an extremely important step for the understanding of basic physiological processes in the brain. To date, there is a single report of PARACEST agents that provide responses to this metal ion. The lanthanide ion chelator consisted of the tetraamide derivative of cyclen and four imino(diacetate) moieties that were envisaged to interact with \(\text{Ca}^{2+}\). The corresponding Yb\(^{3+}\) and Eu\(^{3+}\) complexes were shown to provide CEST responses to \(\text{Ca}^{2+}\); however, Mg\(^{2+}\)-induced similar CEST changes.\(^9\)

On the other hand, excellent and selective responses to \(\text{Ca}^{2+}\) were previously obtained for a series of GdDO3A-based mono- and bismacroyclic SCAs.\(^10,11\) In either of the cases, the organic molecule was comprised of two different moieties: a cyclen-based ring(s) appended with acetate arms for Gd\(^{3+}\) chelation and an acyclic EGTA-derived part (EGTA = ethylene glycol-bis(2-aminoethyl ether)-\(N,N,N',N''\)-tetraacetic acid), as a high affinity and selective calcium chelator. The detailed studies on these systems revealed that Ca-induced alteration of the hydration number \(q\) was the major factor responsible for the longitudinal relaxation \(r_1\) change.\(^10\) The \(q\) alteration is the direct consequence of the change in coordination of the carboxylate groups in the EGTA-derived chelator in the major square antiprismatic (SAP) isomer, which flips from the Gd\(^{3+}\) coordination environment to \(\text{Ca}^{2+}\) upon its addition. Moreover, the amide groups of the EGTA-derived chelator are also expected to be in the vicinity of the lanthanide ion in the presence or absence of \(\text{Ca}^{2+}\).\(^11\)

Having these insights on the specific coordination aspects that produce remarkable \(r_1\) changes on Gd-based Ca-responsive SCAs, we sought to investigate their structural analogues that could potentially serve as responsive PARACEST agents. However, polyanimo polycarboxylate DOTA/DO3A-type ligands are not suitable for providing a CEST effect due to the rapid exchange of the coordinated water molecule, hence they should be converted into slow-exchanging species.\(^3\) This can be achieved by replacing the polyanionic arms of the ligand with the neutral ones, thereby decreasing the water exchange rate of the complex and making the agents suitable for PARACEST.\(^12,13\) The most commonly investigated ligands for this purpose are tetraamide derivatives of DOTA, especially DOTAM-gly and its derivatives, although DO3A and DO2A amide derivatives have also been reported.\(^14\) Thus, in an attempt to prepare \(\text{Ca}^{2+}\) responsive PARACEST agents we designed three different bismacroyclic ligands, each of them bearing a standard EGTA-derived Ca-chelator coupled to amide-type macrocyclic chelators for the complexation of lanthanide metal ions. We varied the groups on the amide moieties aiming to investigate the effect of charge, hydrophobicity and steric hindrance. This resulted in the use of glycine, methyl and tert-butyl substituents (L\(^1-3\), respectively) to replace the six acetic moieties of the \(T_1\)-responsive SCA (Chart 1).

Upon their synthesis, various physicochemical aspects were investigated, including their CEST effect, hydration number assessment by means of time-resolved luminescence decay measurements, NMR studies and estimations of stability constants with endogenous metal ions by means of potentiometric titrations. CEST studies and luminescence lifetime measurements in the presence of \(\text{Ca}^{2+}\) were also carried out to assess the responsiveness of the synthesized agents to this metal ion.

Results and discussion

Synthesis of the ligands

The desired ligands were prepared according to a convenient six-step procedure (Scheme 1). The synthesis commenced from the commercially available cyclen, which was monoalkylated with benzyl(3-bromopropyl)carbamate 1 to give the building block 2. The installation of different amide substituents was accomplished by alkylation of 2 with particular halogenides.

![Chart 1 Chemical structures of ligands L\(^1-3\) investigated in this work.](image-url)
3a–c in acetonitrile. The primary amines 5a–c were obtained by reductive hydrogenation of 4a–c in ethanol using 10% Pd on carbon as a catalyst. Further coupling of the obtained amides 6a–c with amine 7 led to the protected bismacroyclic ligands 8a–c, which were treated with hydrochloric or formic acid to afford the desired ligands L1–3. Finally, bimetallic Eu3+ or Yb3+ complexes were prepared by mixing the ligands with the corresponding LnCl3 salt while maintaining the pH value between 6 and 7.

**CEST properties**

All the Z-spectra had been initially acquired at 25 °C with identical concentrations of the complexes (15 mM per Ln3+). Eu₂L₁ exhibited a weak CEST effect at 57 ppm, which is attributed to the proton exchange between the Eu-bound and bulk water. Temperature enhancement to 37 °C had a noticeable influence resulting in an almost double increase in intensity and an upfield shift of 3 ppm (Fig. 1a and b). Previously it was shown that PARACEST agents are more suitable for noninvasive MRI thermometry methods than those depending on T₁ relaxation-time changes, chemical shift or the diffusion coefficient of bulk water. In the former case a strong linear dependence of the chemical shift of the bound water pool with temperature was observed (≈0.5 ppm °C⁻¹), while methods based on the determination of diffusion coefficients present low temperature sensitivity (≈0.01 ppm °C⁻¹). The CEST effect observed for Eu₂L₁ is in good correlation with these findings, showing a shift of 3 ppm for the increase in temperature of 12 °C (≈0.25 ppm °C⁻¹). However, the addition of Ca²⁺ (up to 10 equiv.) did not provoke a marked change in the CEST effect at any of the investigated temperatures. This result suggests that Ca²⁺ addition does not trigger important changes in the coordination environment of the paramagnetic Eu³⁺ centre, unlike the Gd-based Ca-sensitive systems containing acetate pendant arms.

The Z-spectra were also recorded using solutions of the complexes with chelators L₂–₃, which possess a net positive charge. For the ligand with N-methylamide groups, both Eu₂L₂ and Yb₂L₂ complexes showed a very weak CEST effect upon saturation of the resonances corresponding to the coordinated water molecule (≈5% and 1% at 53 ppm and 230 ppm, respectively). Similar to Eu₂L₁, a very small quenching of the CEST

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**Scheme 1** Synthesis of the ligands L₁–₃. Reagents and conditions: (i) Et₃N, CH₂Cl₂, RT, 59%. (ii) 3a–c, K₂CO₃, CH₃CN, 70 °C, 66–83%. (iii) H₂, Pd/C, EtOH, RT, 92–96%. (iv) (ClCH₂CO)₂O, Et₃N, CH₃CN, RT, 74–82%. (v) K₂CO₃, KI, CH₃CN, 70 °C, 67–79%. (vi) HCl or HCOOH, RT, 86–93%.
signal was observed upon Ca\(^{2+}\) addition in both of these complexes (Fig. 1c and d). Moreover, the Yb\(_2\)L\(_2\) complex provides a weak CEST effect at an offset frequency of \(\sim -24\) ppm that remains nearly unaffected upon addition of Ca\(^{2+}\). This CEST effect is attributed to exchangeable NH protons, and is in agreement with previous findings that revealed CEST peaks due to amide protons in Yb\(^{3+}\) complexes in the range \(-15\) to \(-29\) ppm.\(^{16-18}\) Finally, the Z-spectra of the complexes with bulky tert-butyl substituents Eu\(_2\)L\(_2\) and Yb\(_2\)L\(_2\) did not show any CEST effect in the presence or absence of Ca\(^{2+}\) (data not shown).

Apparently, the acquired Z-spectra and obtained CEST properties indicated that the prepared DO3A-amide Eu\(^{3+}\) and Yb\(^{3+}\) complexes likely have different coordination characteristics from their carboxylic Gd\(^{3+}\) analogues. The strongest CEST signal was obtained for the DO3AM-gly-type derivative (Eu\(_2\)L\(_1\)), suggesting that the polarity of the side arms and the overall charge of the complexes play important roles in determining the exchange rate of the coordinated water molecule responsible for the CEST signal. Previous reports showed that the introduction of bulky groups into the amide side arms of DOTAM derivatives accelerates water exchange in a favourable way for the CEST effect,\(^{19}\) although the opposite effects were also obtained.\(^{20}\) In this study the CEST effect gradually decreases on the DO3AM-type bismacroyclic derivatives towards the less polar and bulky substituents resulting in the preferred order CH\(_2\)COOH > CH\(_3\) > C(CH\(_3\))\(_3\). However, the absence of any response to Ca\(^{2+}\) (its addition did not cause observable reductions or increases in CEST effects on Eu\(_2\)L\(_1\)–2 or Yb\(_2\)L\(_2\)) suggested that the coordination environment of the paramagnetic ions in these triamide systems has been changed compared to their tricarboxylic analogues, requiring further investigation.

**Luminescence experiments**

The hydration states in the presence and absence of Ca\(^{2+}\) can provide a good indication of potential changes in the environment of the paramagnetic ion upon Ca\(^{2+}\) addition. Thus, the luminescence emission lifetimes of Eu\(_2\)L\(_1\)–3 were recorded in H\(_2\)O and D\(_2\)O, and the hydration numbers \(\langle q \rangle\) were calculated.

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**Fig. 1** The CEST spectra of 15 mM complexes (irradiation time 3 s, \(B_1 = 25\) \(\mu\)T) recorded in the absence (red) and presence (black) of 1 equiv. of Ca\(^{2+}\) for: (a) Eu\(_2\)L\(_1\), recorded at 25 and 37 °C, showing signals at 57 ppm and 54 ppm, respectively; (b) Eu\(_2\)L\(_1\) (magnified CEST effects, conditions provided under (a); (c) Yb\(_2\)L\(_2\), insets show magnified signals originating from bound water (230 ppm) and amide (\(\sim -24\) ppm) protons; (d) Eu\(_2\)L\(_2\) the inset shows the magnified signal originating from bound water (53 ppm).
The obtained results show that the q number stays constant, within the experimental error, upon calcium addition to all the three investigated complexes. This behaviour is opposite to that of DO3A-based analogue SCA systems, which showed an increase in the number of inner-sphere water molecules upon Ca\(^{2+}\) addition.\(^{10,11}\) Further, the findings indicate that Eu\(_2\)L\(^1\) and Eu\(_2\)L\(^2\) are monohydrated complexes, while the less sterically hindered complex Eu\(_2\)L\(^3\) displays an equilibrium between dihydrated and monohydrated species. The absence of prominent CEST effects upon Ca\(^{2+}\) addition to aqueous solutions of Eu\(_2\)L\(^1-3\) can certainly be correlated with hydration states remaining constant, indicating that the addition of Ca\(^{2+}\) does not provoke significant changes in the coordination environment of the lanthanide ion.

The luminescence steady-state emission spectra of Eu\(_2\)L\(^1-3\) in H\(_2\)O were also recorded. Similarly to the decay experiments, addition of Ca\(^{2+}\) did not produce any significant change in the intensity or shape of the major \( \Delta 7F_0 \rightarrow \Delta 5D_0 \) transitions (Fig. S1 in the ESI\(^\dagger\)). However, further splitting of the signals due to the \( \Delta 5D_0 \rightarrow \Delta 7F_1 \) and \( \Delta 5D_0 \rightarrow \Delta 7F_4 \) transitions and change in the intensity ratios of \( \Delta 5D_0 \rightarrow \Delta 7F_1 \) and \( \Delta 5D_0 \rightarrow \Delta 7F_4 \) transitions at 590 and 615 nm were observed at basic pH for Eu\(_2\)L\(^1\)-2, suggesting the change in polarisability of the axial donor and the local symmetry at the metal centre (Fig. S2 in the ESI\(^\dagger\)). The major cause for these spectral alterations was apparently a change in protonation states of amines from the EGTA-derived chelator and inner-sphere water molecules (see below).

### NMR studies

The \(^1H\) NMR spectra of Eu\(_2\)L\(^1-3\) complexes were recorded in D\(_2\)O solutions at pH \( \sim 8.0 \) (Fig. 2). They present relatively broad resonances that spread over the range \( \sim 20 \) to 30 ppm due to the paramagnetic shifts induced by the metal ion. The spectrum of Eu\(_2\)L\(^1\) points to the presence of at least three different isomers in solution. It is well known that DOTAs-like complexes may exist in solution as two different isomers providing either a square-antiprismatic (SAP) or a twisted-square antiprismatic (TSAP) coordination around the lanthanide(m) ion. These isomers differ either in the orientation of the pendant arms of the macrocycle, which is often denoted as \( \Delta \) or \( \Lambda \), or the conformation of the cyclen moiety ([\( \delta \delta \delta \delta \delta \)] or [\( \lambda \lambda \lambda \lambda \lambda \)]).\(^{22,23}\) In Eu\(^{3+}\) complexes of DOTA and DO3A derivatives the signals of the pseudo-axial protons on the cyclen rings are usually found between 24 and 45 ppm in the square antipris-
omitted for simplicity), and the corresponding cumulative stability constants provided in eqn (2).

\[ p\text{Eu} + q\text{H}^+ + r\text{L} \rightleftharpoons \text{Eu}_q\text{H}_r\text{L}_p, \quad \beta_{p,q,r} \quad (L = L^1 \text{ or } L^2) \]  

(1)

\[
\beta_{p,q,r} = \frac{[\text{Eu}_p\text{H}_q\text{L}_r]}{[\text{Eu}^2]^p[H]^q[L]^r} \]  

(2)

In order to study speciation in the three-component systems \(\text{Eu}^3+–\text{H}^+–\text{L}^\) or \(\text{Eu}^3+–\text{OH}^––\text{L}^\), it was necessary to characterize the binary equilibria, i.e., hydrolysis of \(\text{Eu}^3+\) and the ligands’ protolytic equilibria. The equilibrium constants of \(\text{Eu}^3+\) hydrolysis were taken from the literature, and the ligand protonation constants calculated using the ADMET Predictor software (Table S1 in the ESI†), showing good agreement with previously published values for DOTAM-type systems. The equilibrium constants of the complexes were determined using the Hyperquad 2008 software (using ionic product value \(K_w = 13.77\)). Species distribution diagrams were plotted according to calculated constants using the HySS software.

The equilibrium constants of \(\text{Eu}^3+–\text{H}^+–\text{L}^\) complexes were determined by acid–base potentiometric titrations (Tables 2 and S2 in the ESI†). Analysis of the potentiometric titration data was performed to find the model that gives the best fit to the experimental data (statistical parameters which determine the quality of fit are provided in Table S2 in the ESI†). The calculations revealed the formation of \([\text{Eu}_n\text{H}_m\text{L}]^z\) \((n = 1, 2, 3)\) as well as \([\text{Eu}_2\text{L}]\) complexes. The formation of \([\text{Eu}_2\text{L(OH)}_2]\) complexes was also noticed. The obtained protonation constants were compared to those previously published for ethylenediaminetetraacetic acid (EDTA), EGTA and EGTA–bisamide (Table 2), while the experimentally determined stability constants with standard deviations are provided in the ESI (Table S2†).

The first two protonation constants \((\log K^1_q \text{ and } \log K^2_q)\) stand for protonation of the amine nitrogen atoms of the EGTA-derived part. These values are quite different for \(\text{Eu}_q\text{L}_1^\) and \(\text{Eu}_q\text{L}_2^\). The complex \(\text{Eu}_q\text{L}_1^\) displays a higher basicity similar to EDTA and especially EGTA; this can be explained by the overall charge as the presence of six carboxylate groups on the macrocycles in \(\text{Eu}_q\text{L}_1^\) neutralize the positive charge of two bound \(\text{Eu}^3+\) ions. This can also be the reason for the relatively high log \(K^q_1\). On the other hand, the highly positively charged \(\text{Eu}_q\text{L}_2^\) behaves similar to the EGTA–bisamide, having one neutral and one acidic amine nitrogen, respectively, and a lower log \(K^q_2\) value compared with \(\text{Eu}_q\text{L}_1^\). Distribution diagrams of species in the \(\text{Eu}_q\text{L}_1^\) and \(\text{Eu}_q\text{L}_2^\) systems, for the concentration ratio \([\text{L}] : [\text{Eu}] = 1 : 2\), indicate this different behaviour (Fig. 3). The equilibrium constants for \(\text{Eu}^3+\) hydrolysis were taken from the literature, and the corresponding cumulative stability constant is given by eqn (4).

\[ p\text{Eu} + q\text{H}^+ + r\text{L} + mM \rightleftharpoons \text{Eu}_{q+r}\text{H}_{q+r}\text{L}_r\text{M}_m, \quad \beta_{p,q,r,m} \]  

(3)

\[
\beta_{p,q,r,m} = \frac{[\text{Eu}_p\text{H}_q\text{L}_r\text{M}_m]}{[\text{Eu}^2]^p[H]^q[L]^r[M]^m} \]  

(4)

These stability constants were also determined by acid–base potentiometric titrations. The obtained values were compared with those previously reported for EDTA, 2,2′-oxybis-(ethyl-

Table 2  Experimentally determined protonation constants of \(\text{Eu}_q\text{L}_1^\)–\(2\) complexes compared to EDTA, EGTA and EGTA–bisamide protonation constants reported in the literature

<table>
<thead>
<tr>
<th>(\text{Eu}_q\text{L}_1)</th>
<th>(\text{Eu}_q\text{L}_2)</th>
<th>EDTA</th>
<th>EGTA</th>
<th>EGTA–bisamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\log K^1_q)</td>
<td>8.72</td>
<td>7.34</td>
<td>10.17</td>
<td>9.47</td>
</tr>
<tr>
<td>(\log K^2_q)</td>
<td>8.29</td>
<td>5.20</td>
<td>6.11</td>
<td>8.85</td>
</tr>
<tr>
<td>(\log K^3_q)</td>
<td>6.82</td>
<td>4.43</td>
<td>2.68</td>
<td>2.26</td>
</tr>
<tr>
<td>(\log K^4_q)</td>
<td>6.95</td>
<td>9.09</td>
<td>2.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

\(^a I = 0.1 \text{ M (NaCl), } t = 25 \pm 1 ^\circ\text{C.} \quad ^b I = 0.1 \text{ M (KCl), ref. 35.}\)
amino)-N,N′,N″,N‴-tetraacetic acid (OBETA) and EGTA (Table 3), while the final set of complexes existing in the studied aqueous solutions and experimentally determined stability constants with standard deviations and statistical parameters showing the quality of fit are provided in the ESI (Table S3†). Finally, the corresponding distribution diagrams of the species in the Eu³⁺–L–M systems (L = L¹ or L² and M = Ca²⁺, Mg²⁺ or Zn²⁺) for the concentration ratio [Eu³⁺]:[L] : [M] = 2 : 1 : 1 are also plotted (Fig. 4 and S6–10 in the ESI†).

The stability constants obtained for Eu₂L¹,² with the investigated metal ions were several orders of magnitude lower than that for the well-studied EDTA, OBETA or EGTA chelators with the same metals. There might be a few reasons for such behaviour. First, the binding affinity of bisamide–bisacid chelators (Eu₂L¹,²) is expected to be weaker than in the case of tetraacetic chelators (EDTA, OBETA or EGTA) due to the reduction in the number of negatively charged carboxylate chelating groups. Next, the additional positive charge on the macrocyclic ring (especially valid for Eu₂L²) induces the repulsion between the Eu₂L¹,² complex and the positively charged metal complex.

This phenomenon can also be confirmed by the differences in stability constants between Eu₂L¹ and Eu₂L², where the former exhibits slightly higher values for all three metal ions due to its lower net positive charge when compared to the latter complex. Finally, the reduced flexibility of the EGTA-derived chelator in Eu₂L¹,² due to the presence of bulky appended macrocycles may prevent efficient wrapping around the metal ion compared with the case of flexible EDTA, OBETA or EGTA chelators.

Furthermore, the obtained stability constants also indicate a decrease in the selectivity of Eu₂L¹,² for Ca²⁺, Mg²⁺ and Zn²⁺. This phenomenon can be easily followed by comparing the ratio of stability constants for a single chelator with two different metals (Table 3). As it can be seen, the ratio of log K_{Eu₂CaL} / log K_{Eu₂MgL} or log K_{Eu₂ZnL} / log K_{Eu₂CaL} for both Eu₂L¹,² is comparable to analogous ratios obtained for EDTA and OBETA despite much lower log K_{MgL} values for Eu₂L¹,² and the structural similarity of the chelating site to EGTA. This loss of selectivity of Eu₂L¹,² for Ca²⁺ vs. Mg²⁺ could be explained by the same reasons that lead to the drop in log K_{MgL} values. Namely, the reduced flexibility of Eu₂L¹,² compared to EGTA prevents better recognition and size match of the EGTA-derived chelator with Ca²⁺ than with Mg²⁺, while the increase in the positive charge of Eu₂L¹,² additionally impairs binding to metal ions and their recognition.

Despite the considerably lower stability constant values for the three investigated metals, the distribution diagrams indicate that heteronuclear complexes are the major species at the physiological pH (Fig. 4 and S6–10 in the ESI†). The heteronuclear complexes [Eu₂(H₃L¹)Ca] (n = 0, 1 or 2) are probably formed by the following reaction:

\[
\begin{align*}
[\text{Eu}_2(H_3L^1)] + \text{Ca}^{2+} & \rightleftharpoons [\text{Eu}_2(H_3L^1)\text{Ca}] ; n = 0, 1 \text{ or } 2.
\end{align*}
\]

Consequently, the dominating complex at lower pH values is [Eu₂(H₃L¹)], with a maximal concentration at pH around 4.5 (Fig. 4). Similar assumptions can be made according to the obtained distribution diagrams of other heteronuclear complexes, [Eu₂(H₃L¹)Mg] and [Eu₂(H₃L¹)Zn] (Fig. S6 and S7 in the ESI†). The protonated heteronuclear complex, [Eu₂(H₃L¹)Ca] has a maximum concentration at pH around 7 and is fairly stable (Table S3 in the ESI†). The formation of the complex [Eu₂(HL¹)Ca] starts at around pH 6 and reaches the maximum concentration at pH 8.2. The complex [Eu₂(L¹)Ca] starts to form at pH 7 and reaches the maximum concentration around pH 9.

**Conclusions**

In this work we synthesised three different bismacroyclic DO3A-amide derivatives appended with the EGTA-derived chelator. The amides had glycine, methyl and tert-butyl as substituents resulting in ligands L¹,³–5, respectively. The paramagnetic complexes of L¹,³–5 were prepared and various aspects of their physicochemical behaviour were investigated. Eu₂L¹ exhibited a greater CEST effect than Eu₂L² and Yb₂L².
complexes, while $\text{Eu}_2\text{L}^1$ and $\text{Yb}_2\text{L}^1$ showed the absence of any CEST effect, suggesting that the polarity of substituents and the overall charge of the complexes play an essential role in the existence of the CEST effect. Addition of $\text{Ca}^{2+}$ led to negligible changes in the CEST effects in the investigated complexes. The luminescence steady-state emission and lifetime measurements confirmed the insensitivity of $\text{Eu}_2\text{L}^1$–3 towards $\text{Ca}^{2+}$ as the inner-sphere hydration of the complexes remained intact in the absence and presence of $\text{Ca}^{2+}$. These findings were in line with the results from $^1\text{H}$ NMR experiments which indicated the presence of different mono- or bis-hydrated isomers of $\text{Eu}_2\text{L}^1$–3 in solution. However, the analysis of NMR data revealed a switch of the SAP/TSAp isomer population for DO3A-amide complexes when compared to the previously investigated responsive Gd-D03A complexes. In DO3A-amides, the TSAp isomer appeared to be the major species, which does not change the coordination environment around the $\text{Ln}^{3+}$ ion (i.e. inner-sphere hydration) upon complexation with $\text{Ca}^{2+}$. It has been shown that the population of the TSAp isomer increases upon increasing the ionic strength for the coordination of the pendant arms to the lanthanide(m) ion. Thus, it is likely that the presence of the amide substituents in $\text{Eu}_2\text{L}^1$–3 introduces some steric hindrance that favors the TSAp isomer over the SAP one. Finally, the results from potentiometric titrations showed a great dependence of protonation constants on the net charge of the complexes $\text{Eu}_2\text{L}^1$–2. They bind $\text{Ca}^{2+}$ with reduced affinity and with a lower selectivity towards $\text{Mg}^{2+}$ or $\text{Zn}^{2+}$ compared to the previously described monoalkylation of cyclen, using 1 as the alkylating agent.

**General remarks**

Commercially available reagents and solvents were used without further purification. Compounds $1, 3a, 3b, 3c, 4a$–$4c$ and $7$ were synthesised according to previously published procedures. Purification of the synthesised compounds was performed using silica gel 60 (0.03–0.2 mm) from Carl Roth (Germany). Standard 0.1 M HCl and NaOH solutions were prepared from ampoules (Titrisol, Merck, Darmstadt, Germany) and potentiometrically standardised. Standard 5 mM $\text{Ca}^{2+}$, $\text{Mg}^{2+}$, and $\text{Zn}^{2+}$ solutions were prepared from appropriate salts and standardised by titration with an EDTA solution. Acros Organics buffers were used for electrode calibration (phthalate pH 4.00, phosphate pH 7.00, and carbonate pH 10.00). All NMR spectra were acquired on a Bruker Avance III 300 MHz, processed using TopSpin 2.1 (Bruker GmbH), and analysed with TopSpin 2.1 or ACD/SpectManager 9.0 (Advanced Chemistry Development, Inc.). The concentration of the complexes was determined using the bulk magnetic susceptibility shift (BMS). ESI-HRMS were performed on a Bruker BioApex II ESI-FT-ICR, equipped with an Agilent ESI-Source, measured via flow injection analysis. ESI-LRMS were performed on an ion trap SL 1100 system (Agilent, Germany). Luminescence lifetime measurements were performed by using a QuantaMasterTM 3-PH fluorescence spectrometer from Photon Technology International, Inc., (Monmouth Junction, NJ, USA). Potentiometric titrations were performed by using a Metrohm Basic Titrino 794 (Herisau, Switzerland) equipped with an InLab Micro electrode (Mettler-Toledo International Inc., Columbus, Ohio, USA).

**Synthetic procedures.** Benzyl[3-{1,4,7,10-tetraazacyclododecan-1-yl}propyl]carbamate ($2$)

Compound 2 was synthesised according to the previously described procedure for monoalkylation of cyclen, using 1 as the alkylating agent.

**Compound 2:** isolated yield: 59%. $^1\text{H}$ NMR (CDCl$_3$, 300 MHz), $\delta$ (ppm): 7.38–7.27 (m, 5H), 5.06 (s, 2H), 3.24–3.18 (br, 2H), 2.73–2.67 (br, 4H), 2.65–2.59 (br, 4H), 2.54–2.40 (br, 10H), 1.72–1.63 (m, 2H). $^{13}\text{C}$ NMR (CDCl$_3$, 75 MHz), $\delta$ (ppm): 156.6, 136.9, 128.3, 128.0, 127.8, 66.2, 52.1, 51.5, 47.0, 46.0, 45.1, 39.3, 27.5. ESI-HRMS: for C$_{19}$H$_{33}$N$_5$O$_2$: calc. 364.2707 [M + H$^+$], found 364.2711.

**General procedure for the synthesis of 4a–4c**

3a, 3b or 3c (3.2 equiv.) was added in an already prepared suspension of 2 (1.0 equiv.) and K$_2$CO$_3$ (4.0 equiv.) in anhydrous acetonitrile. The reaction mixture was stirred at 65 °C for 18 h. After cooling, the product mixture was filtered and the solvent was removed under reduced pressure. The residue was dissolved in dichloromethane and washed twice with water. The organic layer was dried over Na$_2$SO$_4$ and then evaporated under reduced pressure. The crude product was purified by column chromatography over silica gel (eluent MeOH in CH$_2$Cl$_2$) to give 4a–c as amorphous solids.

**Compound 4a.** Starting from 3a (4.30 g, 20.7 mmol), 4.05 g of 4a (66%) was obtained. The eluent for column chromatography purification was 7% MeOH in CH$_2$Cl$_2$. $^1\text{H}$ NMR (CDCl$_3$, 300 MHz), $\delta$ (ppm): 7.28–7.22 (m, 5H), 5.00 (s, 2H), 3.84–3.73 (br, 6H), 3.29–3.26 (br, 26H), 1.62–1.52 (m, 2H), 1.37–1.31 (overlapping s, 27H). $^{13}\text{C}$ NMR (CDCl$_3$, 75 MHz), $\delta$ (ppm): 172.2, 171.4, 169.1, 168.9, 168.8, 156.5, 136.9, 128.4, 128.1, 127.9, 81.8, 81.3, 66.2, 57.1, 56.8, 54.1, 53.5, 52.9, 51.1, 49.9, 41.9, 41.7, 38.9, 28.1, 27.9. ESI-HRMS: for C$_{43}$H$_{72}$N$_8$O$_{11}$: calc. 899.5213 [M + Na$^+$], found 899.5219.
Compound 4b. Starting from 3b (5.00 g, 32.9 mmol), 4.40 g of 4b (74%) was obtained. The eluent for column chromatography purification was 30% MeOH in CH₂Cl₂. ¹H NMR (CDCl₃, 300 MHz), δ (ppm): 7.38–7.27 (m, 5H), 7.08 (s, 2H), 3.23–3.17 (m, 2H), 3.03 and 3.02 (overlapping s, 6H), 2.79–2.49 (br, 27H), 1.74–1.64 (m, 2H). ¹³C NMR (CDCl₃, 75 MHz), δ (ppm): 171.6, 171.3, 156.5, 136.5, 128.5, 128.2, 66.6, 59.1, 58.5, 54.0, 53.4 (br), 53.3 (br), 52.8, 39.6, 25.9, 25.8. ESI-HRMS: for C₃₀H₂₄NO₂₂·Na: calc. 577.3820 [M + H]+, found 577.3830.

Compound 4c. Starting from 3c (4.80 g, 24.7 mmol), 4.50 g of 4c (83%) was obtained. The eluent for column chromatography purification was 10% MeOH in CH₂Cl₂. ¹H NMR (CDCl₃, 300 MHz), δ (ppm): 7.28–7.25 (m, 5H), 5.0 (s, 2H), 3.52–2.65 (br, 26H), 1.85–1.61 (m, 2H), 1.29–1.27 (overlapping s, 27H). ¹¹C NMR (CDCl₃, 75 MHz), δ (ppm): 168.6 (br), 168.0, 155.8, 135.7, 127.5, 127.0, 65.5, 56.8 (br), 52.5, 50.6 (br), 48.7 (br), 37.7, 27.7, 27.5 (br). ESI-HRMS: for C₂₇H₆₅NO₃O₂: calc. 703.5 [M + H]+, found 703.5.

General procedure for the synthesis of 5a–5c

10% Pd/C was added to a solution of 4a (3.00 g, 3.4 mmol), 4b (3.70 g, 6.4 mmol) or 4c (3.10 g, 4.4 mmol) in ethanol (50 mL). The resulting mixture was stirred at room temperature under a hydrogen atmosphere (3 bar) for 18 hours. The reaction was then filtered over Celite® to remove the catalyst and the solvent was removed by rotary evaporation. The residue was dissolved in CH₂Cl₂ (50 mL) and the precipitate was removed by filtration. The organic layer was dried over Na₂SO₄ and then evaporated to give 5a (2.40 g, 94%), 5b (2.60 g, 92%) or 5c (2.40 g, 96%).

Compound 5a. ¹H NMR (CDCl₃, 300 MHz), δ (ppm): 3.77 and 3.76 (overlapping s, 6H), 3.55–2.43 (br, 26H), 1.60–1.47 (m, 2H), 1.39–1.32 (overlapping s, 27H). ¹¹C NMR (CDCl₃, 75 MHz), δ (ppm): 173.1, 169.2, 168.5, 81.7, 81.5, 81.3, 58.4, 57.4 (br), 50.8 (br), 48.7, 42.1 (br), 41.9 (br), 40.0 (br), 28.1 (br), 28.0 (br). ESI-HRMS: for C₂₃H₄₆N₆O₇·Na: calc. 743.5025 [M + H]+, found 743.5025.

Compound 5b. ¹H NMR (CD₃OD, 300 MHz), δ (ppm): 3.38–3.05 (br, 8H), 2.79–2.45 (br, 27H), 1.85–1.70 (m, 2H). ¹¹C NMR (CD₃OD, 75 MHz), δ (ppm): 174.2, 174.0, 173.0, 59.8, 58.9, 52.2 (br), 51.9, 51.7, 40.4, 26.3 (br), 24.9. ESI-HRMS: for C₂₅H₄₄N₆O₇·Na: calc. 443.3453 [M + H]+, found 443.3458.

Compound 5c. ¹H NMR (CDCl₃, 300 MHz), δ (ppm): 3.61–2.40 (br, 26H), 1.94–1.55 (m, 2H), 1.38–1.34 (overlapping s, 27H). ¹¹C NMR (CDCl₃, 75 MHz), δ (ppm): 171.2, 170.8, 169.9, 169.7, 66.4 (br), 58.0, 51.8, 51.7 (br), 51.6 (br), 51.5 (br), 50.7 (br), 28.8 (br), 28.5 (br). ESI-HRMS: for C₂₅H₄₆N₆O₇·Na: calc. 569.4861 [M + H]+, found 569.4866.

General procedure for the synthesis of 6a–6c

5a, 5b or 5c (1 equiv.) and Et₃N (1.5 equiv.) were dissolved in acetonitrile and the reaction mixture was heated at 70 °C overnight under a nitrogen atmosphere. The inorganic salts were removed by filtration and the solvent was evaporated to dryness. The crude product was purified by column chromatography over silica gel using 8% MeOH in CH₂Cl₂ as the eluent to give 8a, 8b or 8c.

Compound 6a. Starting from 6a (1.30 g, 1.6 mmol), 0.90 g of 8a (70%) was obtained. ¹H NMR (CDCl₃, 300 MHz), δ (ppm): 8.44 (s, 3H), 7.93 (s, 2H), 7.55 (s, 3H), 3.76 and 3.75 (overlapping s, 12H), 3.69–3.53 (br, 12H), 3.43 (s, 4H), 3.33–3.20 (br, 14H), 2.91–2.30 (br, 42H), 1.58–1.55 (m, 4H), 1.38–1.34 (overlapping s, 72H). ¹¹C NMR (CDCl₃, 75 MHz), δ (ppm): 172.6, 171.1 (br), 169.9, 168.9, 168.4, 82.4, 81.9, 81.6, 69.6, 68.4, 58.7 (br), 57.7 (br), 51.1 (br), 50.9 (br), 50.6, 50.3, 48.5, 41.8 (br), 41.7 (br), 39.9 (br), 29.6 (br), 27.9 (br), 23.8 (br).

Compound 6b. Starting from 6b (1.50 g, 2.9 mmol), 1.09 g of 8b (67%) was obtained. ¹H NMR (CD₃OD, 300 MHz), δ (ppm): 3.89–2.4 (br, 90H), 2.14–2.04 (m, 2H), 1.84–1.74 (m, 2H), 1.55–1.52 (overlapping s, 18H). ¹¹C NMR (CD₃OD, 75 MHz), δ (ppm): 175.1 (br), 174.5 (br), 174.0 (br), 173.5, 173.0, 168.8, 84.8, 82.9, 71.3, 71.1, 70.2, 67.9, 60.0, 58.6 (br), 58.1 (br), 56.0, 28.6, 28.5, 26.7, 26.5.
Compound 8c. Starting from 6c (1.20 g, 1.9 mmol), 0.98 g of 8c (79%) was obtained. 1H NMR (CDCl3, 300 MHz), δ (ppm): 3.85–2.30 (br, 72H), 1.90–1.80 (m, 4H), 1.51–1.26 (overlapping s, 72H). 13C NMR (CDCl3, 75 MHz), δ (ppm): 169.4, 166.5, 162.8, 80.8, 67.0, 53.4, 50.1 (br), 48.6, 48.2 (br), 46.9, 44.5, 26.9 (br), 26.7 (br), 26.6 (br), 26.1 (br).

General procedure for the synthesis of L1–3
Bismacrocycle 8a, 8b or 8c was dissolved in formic acid (3 mL) and the solution was heated at 60 °C for 18 h (for potentiometric titrations, L1–2) were prepared by mixing 8a,b with 4 N HCl (3 mL) in dioxane at RT for 4 hours. After the solution was cooled, formic acid was removed on the rotary evaporator. The residue was dissolved in a minimal amount of water, added dropwise to cooled acetone (–20 °C) and stored in the freezer overnight. The acetonite was decanted from the solid material and the crude products L1–3 were obtained by drying in a vacuum.

Ligand L1. Starting from 8a (0.70 g, 0.4 mmol), 0.48 g of L1 (89%) was obtained. 1H NMR (D2O, 300 MHz), δ (ppm): 4.08 (s br, 4H), 3.89–3.72 (br, 16H), 3.69 (s, 2H), 3.63–3.59 (br, 6H), 3.50–2.98 (br, 56H), 2.14–2.04 (m, 4H). 13C NMR (D2O, 75 MHz), δ (ppm): 175.62, 175.20, 172.11 (br), 171.03, 69.62, 65.51, 55.49, 55.17, 51.64 (br), 51.30, 49.03 (br), 46.75, 42.82, 36.59, 30.50, 31.37.

Ligand L2. Starting from 8b (0.45 g, 0.3 mmol), 0.38 g of L2 (93%) was obtained. 1H NMR (CD3OD, 300 MHz), δ (ppm): 3.74–3.20 (br, 52H), 3.03–2.69 (br, 38H), 2.00–1.90 (m, 4H). 13C NMR (CD3OD, 75 MHz), δ (ppm): 173.58, 171.00, 167.59, 164.21, 162.8, 80.8, 70.8, 67.0, 53.4, 50.1 (br), 48.6, 48.2 (br), 46.9, 44.5, 26.9 (br), 26.7 (br), 26.6 (br), 26.1 (br).

Ligand L3. Starting from 8c (0.66 g, 0.4 mmol), 0.53 g of L3 (86%) was obtained. 1H NMR (CD3OD, 300 MHz), δ (ppm): 3.95–2.61 (br, 72H), 1.92–1.80 (m, 4H), 1.26–1.13 (overlapping s, 54H). 13C NMR (CD3OD, 75 MHz), δ (ppm): 171.10, 167.00, 167.41, 71.32, 67.16, 62.64, 55.77, 53.04 (br), 52.28 (br), 50.44 (br), 29.26 (br), 29.21 (br), 29.08.

General procedure for the preparation of Ln3+ complexes
The Ln3+ complexes of L2 and L3 were prepared by mixing the respective ligand and the LnCl3 solutions in a 2:1 (Ln3+/L) molar ratio. The exact amount of ligand was determined by elemental analysis. The solution was stirred at RT for 48 h. The pH value was adjusted to 7.0–7.5 using 1 M NaOH. The absence of a free ion (Yb3+ or Eu3+) was verified by colorimetric assay using xylene orange.

In the case of negatively charged L3, EuCl3 was added in slight excess (>2 equiv.). The mixture was stirred for 48 h at RT maintaining the pH at 7.0–7.5, using 1 M NaOH. The resulting solution was treated with Chelex 100 to remove the excess Eu3+ and the absence of free Eu3+ was verified by colorimetric assay using xylene orange.

Luminescence lifetime experiments
The lifetime measurements were performed on a QuantaMasterTM 3 PH fluorosence spectrometer from Photon Technology International, Inc. The measurements were performed in H2O and D2O (25 °C) at 5 mM Eu3+ concentration. The Eu3+ ion was directly excited at 395 nm, and the emission intensity at 615 nm was measured with a 10 µs resolution. The excitation and emission slits were set to 15 and 5 nm bandpass, respectively. Data sets are an average of 25 scans, and each reported value is the mean of three independent measurements. The obtained curves are fitted to a first-order exponential decay with r2 = 0.99. The q values were calculated using eqn (5).13

Potentiometric titrations
Experiments were performed at t = 25 ± 1 °C, with a constant argon flow, using a 794 Basic Titrino automatic titrator (Metrohm, Herisau, Switzerland) equipped with an InLab Micro electrode (Mettler-Toledo International Inc., Columbus, Ohio, USA). The electrode–pH-meter system was calibrated by means of a strong acid–strong base titration in 0.1 M NaCl, using GLEE – GLass Electrode Evaluation software;44 standard potential (E0 = 383.6 ± 0.2 mV) and slope (57.4 ± 0.1 mV) are obtained as mean values of four titrations. Hyperquad 2008 software was used to calculate protonation and stability constant values as the mean values of four titrations.13

Eu3L (L = L1 or L2) protonation constant determination.
Eu3L complexes were previously synthesized in a solution. The concentration of stock Eu3L solutions was determined according to the BMS method.42 Stock Eu3L solutions were diluted with 0.1 M NaCl to prepare working solutions [Eu3L] = 2.4547 × 10–4 M, [Eu3L2] = 4.9971 × 10–4 M). Prior to titration, standard HCl solution (0.0984 M; 35.0 µL for Eu3L1, and 60.0 µL for Eu3L2) was added to 4.00 mL of Eu3L working solutions to reach pH 4. All probes were titrated with 2.0 µL increments of standard NaOH solution (0.1008 M) in the 4.0–10.0 pH range.

Eu3L (L = L1 or L2) stability constants with Ca2+, Mg2+ and Zn2+ determination. Prior to titration, standard HCl solution (0.0984 M; 35.0 µL for Eu3L1, and 60.0 µL for Eu3L2) and 1.0–1.1, 1.2–1.3, and 1.5–1.6 mol-equivalents of standard Ca2+, Mg2+, or Zn2+ solutions were added to 4.00 mL of Eu3L working solutions and stirred for 10 minutes. All probes were then titrated with 2.0 µL increments of standard NaOH solution (0.1008 M) in the 4.0–10.0 pH range. Total concentrations of the ligand and metal ion used in each experiment are provided in the ESI.†

Acknowledgements
The financial support of the Max-Planck Society and the European COST CM1006 Action is gratefully acknowledged.
Notes and references


