Ln(III)-complexes of a DOTA analogue with an ethylenediamine pendant arm as pH-responsive PARACEST contrast agents†

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A novel macrocyclic DO3A derivative containing a linear diamine pendant arm, H3do3aNN, was prepared and its protonation and complexation properties were studied by means of potentiometry. It determined ligand consecutive protonation constants \( \log K_{A1} = 12.62 \), \( \log K_{A2} = 10.28 \), \( \log K_{A3} = 9.67 \), \( \log K_{A4} = 8.30 \), \( \log K_{A5} = 3.30 \) and \( \log K_{A6} = 1.58 \) and stability constants of selected lanthanide (Eu(III), Yb(III)) complexes \( \log K_{EL} \approx 23.16 \) and \( \log K_{CL} \approx 22.76 \). The complexes could be protonated on the pendant amino group(s) with \( \log \Delta K_{HLM} \approx 5.6 \) and \( \log \Delta K_{(H2LM)} \approx 4.8 \). Solution structures of both complexes were studied by NMR spectroscopy. The study revealed that the complex species exist exclusively in the form of twisted-square-antiprismatic (TSA) isomers. The complexes show significant pH dependence of the Chemical Exchange Saturation Transfer (CEST) between their amino groups and the bulk water molecules in the pH range of 5–8. Thus, the pH dependence of the magnetization transfer ratio of CEST signals can be used for pH determination using magnetic resonance imaging techniques in a pH range relevant for in vivo conditions.

Introduction

Magnetic resonance imaging (MRI), due to its non-invasive character and spatial resolution (down to a mm³ at clinical magnetic fields), is currently one of the most important diagnostic methods used in clinical medicine. Relevant diagnostic information from MRI images can be obtained even with the natural contrast between various tissues. However, for further improvement of image contrast and resolution, exogenous contrast agents (CAs) based on complexes of highly paramagnetic metal ions or superparamagnetic nanocrystalline materials altering the relaxation times of bulk water are widely used.1 In addition to common MRI \( T_1 \)- and \( T_2 \)-contrast agents, which shorten the longitudinal (\( T_1 \)) and transversal (\( T_2 \)) relaxation times,2 a new class of CAs based on a Chemical Exchange Saturation Transfer (CEST) mechanism was introduced in the past decade.3

The principle of the CEST effect is based on saturation of the proton signal of the contrast agent molecule by a selective rf-pulse. This saturation is transferred to the surrounding water molecules via chemical exchange of the labile protons between the contrast agent and bulk water resulting in a decrease in the water signal intensity and, therefore, darkening of the corresponding area in the MR image. To reduce any nonspecific water proton irradiation and to increase the sensitivity of the CEST CAs, paramagnetic complexes (PARACEST agents) are used to shift the resonance frequency of the exchangeable protons far away from that of bulk water.4,5 These agents contain a paramagnetic metal ion chelated by a multidentate ligand. Most often, Ln(n) complexes of ligands derived from DOTA (thus ensuring high stability and kinetic inertness of the complexes) have been used.6,7 As alternatives, complexes of transition metal ions having suitable magnetic properties, such as Ni(n), Fe(n) or Co(n), with ligands based on cyclam, cyclen, 1,4,7-triazacyclononane or 1,4,10-trioxa-7,13-diazacyclopenta-decane, etc. have also been reported (Fig. 1).8

One of the major advantages of CEST agents is the possibility to modulate water signal intensity by a selective pre-saturation pulse and, therefore, image contrast produced by these CAs can be switched “on” or “off” at will by selecting the appropriate irradiation frequency. This fact makes it possible to detect several agents in the same sample.9 Another advan-
tage lies in the sensitivity of the proton exchange rate \( k_{\text{ex}} \) to a number of external factors and, thus, the CEST complexes are suitable for measuring various physiological parameters, such as temperature, pH, metabolite or metal ion concentration, etc.\(^4\)

Recently, a lot of effort has been invested into developing MRI CAs capable of reporting on in vivo changes of pH in a tissue as they could serve as valuable biomarkers of disease progression or indicators for the choice of treatment.\(^9\) Several studies have demonstrated the unique ability of PARACEST CAs to act as pH sensors and nowadays ratiometric methods are being explored to make the assessments independent of the local concentration of the CAs.\(^11\) For example, a Yb(III) analogue of the clinically approved MRI CA [Gd(do3a-hp)] (ProHance\textsuperscript{®}, ligand shown in Fig. 1) shows two independent well-resolved PARACEST peaks at 71 and 99 ppm originating from the protons of the coordinated alcohol group of individual complex isomers.\(^11\) The ratio of these two PARACEST signals is pH-dependent, which can be used to develop a concentration-independent method of pH measurement, and the Yb(III) complex has been already tested for measuring extracellular pH in murine melanoma.\(^11\)

It was shown that Ln(III) complexes of cyclen derivatives with pendant arms containing an amido-amine pendant arm,\(^11\) or a (semi)coordinating amino group\(^12\) produced a pH-sensitive PARACEST effect in the pH region relevant for living systems. Based on these findings, we decided to synthesize a new macrocyclic ligand H\(_3\)do3aNN (Fig. 1) containing a semi-labile coordinating pendant arm with two (primary and secondary) amino groups (as two potentially independent proton exchanging pools), and to investigate the PARACEST properties of its Ln(III) complexes.

**Results and discussion**

**Synthesis**

The synthesis of H\(_3\)do3aNN is shown in Scheme 1. The alkylation agent 3 was prepared by CBr\(_4\)/PPh\(_3\) bromination of ethyl-carbamate-protected N-(2-aminooethyl)ethanolamine.\(^2\) The alkylation of \(\text{tBu}_3\)do3a-HBr was performed using a slight excess of the alkylation agent as, under the reaction conditions, the alkylation agent undergoes elimination of HBr. The \(\text{tBu}\)-ester groups were removed by reflux in a CF\(_3\)CO\(_2\)H : CHCl\(_3\) 1 : 1 mixture and the ethyl-carbamate protection groups were removed by hydrolysis in 10% aq. NaOH. Surprisingly, in this reaction step, preferential formation of the urea-derivative 6 was observed, with only trace amounts of the required compound H\(_3\)do3aNN. However, the intermediate 6 can be isolated by crystallization in a zwitterionic form with 42% overall yield (based on \(\text{tBu}_3\)do3a). The identity of the intermediate 6 was confirmed by a single-crystal X-ray diffraction study (see ESI and Fig. S1\(^\dagger\)).

Hydrolysis of 6 with aq. HCl produced H\(_3\)do3aNN with a high yield. The best way to obtain the product in the solid form was trituration of the evaporated reaction mixture in dry THF or EtOH overnight. However, the resulting off-white solid is very hygroscopic and has to be

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**Fig. 1** Structural formulas of the ligands discussed in the text.

**Scheme 1** Synthesis of H\(_3\)do3aNN. (i) CH\(_3\)CH\(_2\)OC(O)Cl, dioxane : H\(_2\)O (1:1), RT, 2 h; (ii) CBr\(_4\), PPh\(_3\), THF, RT, 1 h; (iii) \(\text{tBu}_3\)do3a-HBr, K\(_2\)CO\(_3\), MeCN, 60 °C, 24 h; (iv) CF\(_3\)CO\(_2\)H : CHCl\(_3\) (1:1), reflux 18 h; (v) 10% aq. NaOH, 90 °C, 24 h; (vi) aq. HCl (1:1), 95 °C, 7 d.
stored in a desiccator over P₂O₅. All other attempts (different organic solvents used for trituration or crystallization) led to the isolation of the title ligand as oil. To prevent possible esterification by EtOH, the use of THF was preferred for trituration.

**Thermodynamic behaviour of H₃do₃aNN and its Ln(III) complexes**

Potentiometric titrations of the ligand performed in the pH range of 1.6–12.2 revealed six consecutive protonation processes in this region (Tables 1 and S1†). Based on comparison with the literature data, the first protonation step (log \( K_\text{p}(\text{HL}) = 12.6 \)) can be attributed to the protonation of one of (or to sharing of a proton over several of) the macrocycle amino groups. The next three protonation steps proceed in part simultaneously due to the similarity of the constants (log \( K_\text{p}(\text{H}_2\text{L}) = 10.3 \), log \( K_\text{p}(\text{H}_3\text{L}) = 9.7 \) and log \( K_\text{p}(\text{H}_4\text{L}) = 8.3 \)) and occur on one other macrocycle amino group and two amino groups of the pendant \( N[2\text{-aminomethyl}]-2\text{-aminomethyl} \) moiety (the value reported for analogous protonation of a 2-aminomethyl pendant moiety for \( \text{H}_3\text{do}_3\text{a-ae} \) is log \( K_\text{p} = 8.9 \)). Further protonations of \( \text{H}_3\text{do}_3\text{aNN} \) proceed on the carbonyl groups and lie in the usual range.

Stability constants of [\( \text{Ln}(\text{do}_3\text{aNN}) \)] (23.16 and 22.76 for the Eu(III) and Yb(III) complexes, respectively, Tables 1 and S2†) were obtained by the out-of-cell titration technique. The values are slightly lower compared with those reported for \( \text{H}_4\text{dota} \) and \( \text{La}(\text{III})/\text{Gd}(\text{III}) \) systems. From these slight differences between the protonation constants, one can conclude that, during out-of-cell titrations, protons in the \([\text{H}_3\text{do}_3\text{aNN}][\text{ML}] \) species occur with log \( p_\text{M} = 22.23 \). The observed values are slightly higher than the protonation constants found for the pre-formed complexes under “non-equilibrium” conditions: in such experiments, complexes were pre-formed at pH ≈ 7 and were titrated employing the standard (“fast”) acid–base titration method. The corresponding observed protonation constants were log \( p_\text{M} = 5.57 \) and 5.67, and log \( p_\text{M} = 4.84 \) and 4.85 for \( \text{La}(\text{III})/\text{Gd}(\text{III}) \) systems.12 From these slight differences between the protonation constants, one can conclude that, during out-of-cell titrations, protons in the \([\text{H}_3\text{do}_3\text{aNN}][\text{ML}] \) and \([\text{H}_3\text{do}_3\text{aNN}][\text{ML}] \) species are probably located not only on the amine pendant arm but, at least

![Equilibrium distribution diagram for the Eu(III)–H₃do₃aNN system](image-url)

**Fig. 2** Distribution diagram of metal-containing species in the Eu(III)–H₃do₃aNN system (c₅₀ = c₁ = 0.004 M, 25 °C, l = 1.0 NMe₄Cl).

**Table 1** Equilibrium constants (log \( K_\text{p} \) and log \( K_\text{st} \)) of H₃do₃aNN (0.1 M NMe₄Cl, 25 °C) and its complexes, and the comparison with corresponding constants reported for related ligands

<table>
<thead>
<tr>
<th>Equilibrium</th>
<th>H₃do₃aNN</th>
<th>H₃do₃a-ae</th>
<th>H₄dota</th>
<th>H₃do₃a-hp</th>
<th>H₃do₃a</th>
<th>H₃do₃a-hp</th>
<th>H₃do₃a-hp</th>
</tr>
</thead>
<tbody>
<tr>
<td>H⁺ + L⁻ ⇌ HL⁻</td>
<td>12.62(2)</td>
<td>10.28(2)</td>
<td>9.67(2)</td>
<td>8.30(3)</td>
<td>3.30(3)</td>
<td>1.58(3)</td>
<td>—</td>
</tr>
<tr>
<td>H⁺ + [Eu(L)]⁺⁻</td>
<td>23.16(5)</td>
<td>6.03(5)</td>
<td>5.09(7)</td>
<td>22.76(4)</td>
<td>6.22(4)</td>
<td>5.07(4)</td>
<td>—</td>
</tr>
<tr>
<td>Gd⁺⁺ + L⁻⁻</td>
<td>23.16(5)</td>
<td>6.03(5)</td>
<td>5.09(7)</td>
<td>22.76(4)</td>
<td>6.22(4)</td>
<td>5.07(4)</td>
<td>—</td>
</tr>
<tr>
<td>[H₃do₃aNN][LaL]</td>
<td>22.23(2)</td>
<td>10.51</td>
<td>4.99</td>
<td>3.84</td>
<td>3.51</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\( p_\text{M} = \log \left[ \frac{[\text{ML}]}{[\text{M}] [\text{L}]} \right] \) for other lanthanides: La(III), \( p_\text{M} = 22.02 \), and Gd(III), \( p_\text{M} = 24.67 \).
partly, also on the macrocycle amino groups. The suggested structures of individual species with tentative protonation sites are shown in Schemes S1 and S2.†

Unfortunately, the [Ln(do3aNN)] complexes are not fully kinetically inert, and slowly dissociate at pH < 6. This was confirmed by a xylene orange test: after the addition of a solution of the pre-formed complex (at pH = 7.5) to a buffered solution of xylene orange at pH = 5.5, the colour gradually changed on standing from orange to orange-violet as a result of free metal appearance in the solution. A quantitative measurement revealed the dissociation of about 9–10% of the complex after standing for one week at room temperature (compare Fig. S16 and S17†). From a thermodynamic point of view, the extent of complex dissociation should be less than 20% at pH = 5 (see the distribution diagram shown in Fig. 2). It was confirmed by independent experiments that neither the free metal aqua ion nor the free ligand interferes with the 1H NMR or CEST measurements. Therefore, conclusions drawn from PARACEST experiments (see below) are fully valid even at pH 5–6.

Solution structure of the [Ln(H\textsubscript{2} do3aNN)]† complexes

It is well-known that in Ln(III) complexes of DOTA-like ligands the central Ln(III) is coordinated between two planes – one formed by the macrocycle amino groups (N\textsubscript{4}-plane), and the other by the oxygen atoms of the carboxylic pendant moieties (O\textsubscript{2}-plane), and these species exhibit two types of isomerisms in solution.\textsuperscript{15} The first type is connected with the conformation of the macrocycle ethylene bridges, i.e. with the sign of the torsion angle around the C–C bond (δ/λ), and the second one is related to the direction of rotation of the pendant arms (∆/Λ). A combination of these isomerisms leads to the formation of two diastereomeric pairs of enantiomers (i.e. four isomers): ∆λλλλ/Λλλλ (SA, square-antiprismatic) and ∆λλλλ/Λλλλ (TSA, twisted-square-antiprismatic).\textsuperscript{2} The isomer ratio in solution can be determined from the 1H NMR spectra using the “axial” protons of the macrocyclic chelate ring, which are the ones closest to the Ln(III) ion and to the principal magnetic axis, and usually can be easily found in the 1H NMR spectra.\textsuperscript{16} Therefore, the solution structures of the [Eu(do3aNN)] and [Yb(do3aNN)] complexes were investigated by variable-temperature 1H NMR spectroscopy (Fig. S2 and S3†). The pD of the samples in D\textsubscript{2}O was adjusted to the alkaline region to ensure full deprotonation and coordination of the pendant amino group. In both complexes, only one set of signals was detected pointing to the presence of only one diastereomer. The signals of “axial” protons appear in the range typical for the TSA isomers (Eu(III): 9–13 ppm, Yb(III): 45–62 ppm; with respect to the signal of bulk water referenced to 0 ppm). No 1H NMR signals of “axial” CH\textsubscript{2} protons attributable to an SA isomer were observed (such signals typically lie in the chemical shift regions of 25–40 ppm and 100–150 ppm for Eu(III) and Yb(III) complexes, respectively).\textsuperscript{16,17} Thus, based on the 1H NMR data, exclusive formation of the TSA isomer is expected. With increasing temperature, the 1H NMR signals become broader, pointing to the occurrence of a conformational change of the complex molecules (Fig. S2 and S3†).

To identify the signals of exchangeable (N–H) protons in the 1H NMR spectra, samples of the [Eu(H\textsubscript{2} do3aNN)] and [Yb(H\textsubscript{2} do3aNN)] complexes were investigated in H\textsubscript{2}O at 25 and 5 °C (Fig. 3, S4 and S5†). In the 1H NMR spectra of the [Eu(H\textsubscript{2} do3aNN)] complex recorded in H\textsubscript{2}O at pH = 6.75 (Fig. 3A), three main signals of exchangeable protons (one narrow signal at 22.2 and two broad signals at 43.3 and 46.5 ppm; with respect to the bulk water signal) can be observed, which disappear upon bulk water presaturation (Fig. 3B). Of these, only the signals at 43.3 and 46.5 ppm are influenced by water presaturation at pH = 11.7, whilst the signal at 22.2 ppm remains unaffected (Fig. 4A and B). When recording 1H NMR spectra in a D\textsubscript{2}O solution (pD = 10.7), none of the three signals are observable (Fig. 4C and S2†). Based on this behaviour, the narrow signal at 22.2 ppm is attributed to the coordinated secondary amino group. The assignment is supported by the similarity of the chemical shift of this signal to that of one of the –NH\textsubscript{2} protons of the [Eu(do3a-ae)] complex (19.5 ppm).\textsuperscript{12} The two broad signals are attributed to the coordinated primary amino group and this assignment is supported by their coalescence at higher temperatures (Fig. S6†).

The primary amino group is expected to be coordinated in a position capping the O\textsubscript{2}N-plane formed by the pendant

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\textsuperscript{†}Such a formula is used when more species differing in protonation are present in a solution. For the range of n refer to the distribution diagram shown in Fig. 2.
donor atoms and, thus, close to the magnetic axis of the complex. Therefore, the corresponding protons are markedly influenced by the paramagnetic ion and their chemical shifts lie in the range typical for a coordinated water molecule.\(^5\),\(^18\) However, the presence of these signals in \(^1\)H NMR spectra also in an alkaline solution (and the presence of a corresponding CEST effect in Z-spectra at alkaline pH, see below) clearly excludes the possibility that these signals belong to a coordinated water molecule, the signal of which disappears in the alkaline region.\(^19\) In slightly acidic solutions where protonation of the uncoordinated primary amino group (and thus, its decoordination) is expected, even a proton of the secondary amino group is exchanged with bulk water on an NMR time scale. In contrast, in an alkaline solution, only the exchange of the terminal primary amino group protons is observable.

Besides the three signals of exchangeable protons of the [Eu(dos3aNN)] complex discussed above, a small signal at \(\approx35\) ppm was found in the \(^1\)H NMR spectra (Fig. 3 and 4), better seen at a lower temperature (37.5 ppm, Fig. S3A\(^\dagger\)).

At this chemical shift, a minor exchangeable pool of protons was found also in the CEST experiments (see discussion of Z-spectra below), accompanied by two other peaks in Z-spectra lying at 10 and 15 ppm, which are visible especially at low saturation power (Fig. 5 and S7A\(^\dagger\)). Due to the absence of any \(^1\)H NMR signals of “axial” \(\text{CH}_2\) protons attributable to the SA isomer, the presence of this isomer in the solution can be excluded. Therefore, these minor exchangeable proton pools were attributed to another TSA isomer that originates from the chirality of the nitrogen atom of the secondary amino group caused by coordination of this group. Judging by the similarity of the chemical shift of the exchangeable proton pool at 35 ppm to one of the signals attributed to the coordinated amino group in [Eu(dos3a-ae)] (34 ppm),\(^12\) one can suggest that this signal belongs to the secondary amino group of the TSA isomer with reverse orientation of \(\text{H}\) vs. the \(\text{CH}_2\text{CH}_2\text{NH}_2\) substituents (i.e. with opposite chirality of the coordinated secondary amine). The results of simple molecular modelling shown in Fig. S9\(^\dagger\) suggest that apical coordination of the primary amino group is possible only in the \(\Delta\delta\delta\delta\delta/S/\Lambda\lambda\lambda\lambda\lambda-R\) enantiomeric pair, and thus, this isomer is suggested to be the major one, leaving the \(\Delta\delta\delta\delta\delta-R/\Lambda\lambda\lambda\lambda\lambda-S\) species as the minor isomer. In the case of this low-abundance isomer, the position of the primary amino group is not suitable for coordination close to the magnetic axis and, therefore, the signals of the primary amino group in Z-spectra are significantly closer (at 10 and 15 ppm, Fig. S7\(^\dagger\)) to the free water signal. As both protons of the primary amino group have individual signals, their resolution triggered by coordination or by fixing in an intramolecular hydrogen bond system is expected.

A similar behaviour was observed also for the [Yb(dos3aNN)] complex. In an alkaline solution, there are two signals disappearing in \(\text{D}_2\text{O}\), see Fig. S4\(^\dagger\) – a narrow signal of the proton of the secondary amino group at 35 ppm (this assignment is supported by the similarity of the chemical shift of the analogous \(^1\)H NMR signal of [Yb(dos3a-ae)], 42 ppm)\(^12\) and a very broad signal of \(\text{NH}_2\) protons at 82–104 ppm (the signals cannot be distinguished at 25 °C, but split at 5 °C, Fig. S5B\(^\dagger\)). As in the previous case, only the signal attributable to the primary...
amino group is affected by water presaturation in an alkaline solution, Fig. S4B†. Minor signals of another TSA isomer are also observable in $^1$H NMR spectra, and minor exchangeable pools of protons were found in CEST experiments at low saturation power (three other peaks in Z-spectra at 17, 26 and 57 ppm, Fig. S7B†).

**CEST experiments**

Saturation transfer experiments in solutions ranging from slightly acidic to slightly alkaline (pH 5.7–8.3) revealed two signals in the $^1$H Z-spectra of each complex. These signals are centred at +22.2 and +44.4 ppm for the Eu(III) complex (Fig. 5A) and +35 and +95 ppm for the Yb(III) complex (Fig. 6A).

The broad signals at the higher chemical shifts (44.4 and 95 ppm for the Eu(III) and Yb(III) complex, respectively) correspond to the averaged signals of the primary amino group. This broad signal splits into two distinct signals of magnetically non-equivalent protons at a lower intensity of presaturation pulses and at low temperatures (Fig. S7A†), similar to the behaviour of this group found in $^1$H NMR spectra (Fig. 3, 4, S5 and S6†). The Z-spectra signals with lower chemical shifts (22.2 and 35 ppm for the Eu(III) and Yb(III) complex, respectively) were attributed to the signal of the proton of the secondary amino group. Thus, the Z-spectra of both complexes clearly confirm the presence of proton-exchanging pools that belong to the protons of the primary and secondary amino groups as they were identified in the $^1$H NMR spectra (see above).

Besides the signals attributable to the major isomer, a set of minor signals (at 10, 15 and 35 ppm) is distinguishable especially when low saturation power was applied) appears in the Z-spectra of the [Eu(H$_n$do3aNN)] complex (Fig. S7A†). At slightly acidic pH, all three Z-signals are apparent. In contrast, in the alkaline region only the signals at 10 and 15 ppm remain in the Z-spectra, implying their assignment to the primary amino group, with the last one (at 35 ppm) belonging to the secondary amino group. These signals belong to the less abundant isomer with opposite chirality of the coordinated secondary amino group (see discussion of $^1$H NMR spectra above). A similar set of minor signals (at 17, 26 and 57 ppm) appears also in the Z-spectra of the [Yb(H$_n$do3aNN)] complex (Fig. S7B†).

The shape of the Z-spectra of the [Ln(H$_n$do3aNN)] complexes (Ln = Eu, Yb) has significant pH dependence in slightly acidic to neutral regions (Fig. 5A and 6A). To see the differences in Z-spectra more clearly, the magnetization transfer ratio (MTR) spectra were constructed (Fig. 5B and 6B). At pH < 5.5, the CEST effect of the coordinated primary amino group gradually disappears as a consequence of protonation and decoordination of the group. Simultaneously, a new, very broad CEST signal appears centred at ≈25–30 ppm for both complexes. Although partial dissociation of the complexes occurs in this pH region (see above for discussion of thermodynamic properties), the free metal aqua ions as well as the free ligand are CEST-silent (as proved by an independent experiment) and, therefore, these new signals can be attributed to the chemical exchange of the protonated primary amino group of the complex. Such a group – whilst uncoordinated – is still paramagnetically shifted, but not as much as when the group is coordinated. On the other hand, an effective CEST of the secondary amino group was detected for the Eu(III) and Yb(III) complexes in the pH region of ≈5.5–8.5. At higher pH values, the chemical exchange of the NH proton becomes too slow to transfer saturation to bulk water and, thus, the CEST effect of the secondary amino group is not observable (Fig. 5A, 6A and S8†). It is consistent with the $^1$H NMR spectra of the studied complexes (Fig. 4 and S4†), where the signals of secondary amino groups are observable even in alkaline solutions.

![Scheme 2](image)

**Scheme 2** A suggested mechanism of origin of pH-dependent CEST effects on [Ln(H$_n$do3aNN)] complexes. In hepta/octa-coordinated species, binding of a water molecule(s) to the central ion giving the coordination number to 8–9 is expected, but it is not shown for the sake of clarity.
Experimental

Materials and methods

All reagents and solvents were commercially available, had synthetic purity and were used as received. Water used for potentiometric titrations was deionized by using a Milli-Q (Millipore).

$^{1,4,7}$-Tris(tert-butylcarboxymethyl)-1,4,7,10-tetraazacyclododecane hydrobromide ($\text{Bu}_3\text{do3a}\cdot\text{HBr}$) was prepared according to the published procedure. THF was dried by the standard method and stored over molecular sieves under an argon atmosphere. Anhydrous MeCN and EtOH were from commercial sources.

NMR characterization data (1D: $^1$H, $^{13}$C{1H}; 2D: HSQC, HMBC, $^1$H–$^1$H COSY) were recorded on a VNMRS300 or Bruker Avance III 600, using 5-mm sample tubes. Chemical shifts are reported as $\delta$ values and are given in ppm. Coupling constants $J$ are reported in Hz. Unless stated otherwise, NMR experiments were performed at 25 °C. For samples dissolved in $\text{D}_2\text{O}$, the pD value was calculated by correcting the pH-electrode reading by +0.4, i.e. pD = pH reading +0.4. For the $^1$H and $^{13}$C

standard deviations of the data points from MRI experiments acquired for the low concentration were relatively high due to a high background noise and, thus, a low signal-to-noise ratio was obtained under these conditions. The final curves are compiled in Fig. 8. Although the method has high ESDs with respect to the determination of an exact pH value, the shape of calibration curves enables distinguishing between samples with pH > 7 and those with pH < 6. Such a finding is relevant for the design of contrast agents useful e.g. for distinguishing between normal and hypoxic tissues.

Fig. 8 Ratiometric plots of the 7.7–8.7 mM [Yb(H$_{12}$do3aNN)] complex, 25 °C; RF presaturation pulse applied for 2 s. Circles: aq. solution, $B_0 = 7.05$ T (NMR), $B_1 = 500$ Hz (11.8 μT) or 920 Hz (21.7 μT). Squares and triangles: 50 mM HEPES–MES, $B_0 = 4.7$ T (MRI), $B_1 = 1060$ Hz (25 μT) or 1490 Hz (35 μT). The ratiometric value (35/95) is the ratio of MTR intensity at 35 ppm to MTR intensity at 95 ppm.
NMR measurements of diamagnetic compounds in D2O, tBuOH was used as an internal standard (δH = 1.25, δC = 30.29). For the measurements in CDCl3, TMS was used as an internal standard (δH = 0.00, δC = 0.00). In the case of paramagnetic complexes, chemical shifts were referred to the water signal of the sample (δH = 0.00) to keep the chemical shift values in 1H NMR spectra consistent with the scale of Z-spectra. The abbreviations s (singlet), t (triplet), q (quartet), m (multiplet) and br (broad) are used in order to express the signal multiplicities. Lanthanide(III) concentrations in solutions were determined by measurement of the bulk magnetic susceptibility (BMS) shifts.22 The ESI-MS spectra were recorded on a Bruker ESI PLUS 3000 spectrometer equipped with an electrospray ion source and ion-trap detection. Measurements were carried out in both the positive and negative modes.

**Synthesis**

**Synthesis of 2.** Ethyl chloroformate (8.02 g, 73.9 mmol, 2.2 eq.) was added dropwise to a well-stirred solution of 1 (3.5 g, 33.6 mmol) in a mixture of dioxane (30 ml) and H2O (30 ml), and the reaction mixture was stirred for 2 h at room temperature. In the next step, conc. aq. NH3 (∼10 ml) was added and the reaction mixture was stirred for 15 min. The mixture was concentrated in vacuo, poured into H2O (50 ml) and extracted with CH2Cl2 (3 × 30 ml). The organic layer was dried over anhydrous Na2SO4 and concentrated in vacuo to yield 4.50 g (54%) of 2 as a colourless oil.

1H NMR (600 MHz, CDCl3): δ 1.15–1.19 (6H, m, CH2CH3); 3.28 (2H, br, NHCH3); 3.36 (4H, br, CH2NCH2); 3.63–3.69 (2H, br, CH2OH); 4.02–4.08 (4H, m, OCH3).

13C{1H} NMR (150.9 MHz, CDCl3): δ 16.14, 16.65 (2C, CH2CH3); 40.03, 40.18 (1C, NHCH3); 48.45, 48.74 (1C, CH2); 51.11, 51.51 (1C, CH3); 60.91 (1C, OCH2); 61.15, 61.40 (1C, CH2OH); 61.66 (1C, OCH3); 157.30 (2C, CO). All four backbone carbon atoms show two 13C NMR signals due to rigid conformations of the molecule locked by different orientations of the amide groups.

MS-ESI: (+) 745.3 ([M + H]+, calcd 745.5); 767.2 ([M + Na]+, calcd 767.5).

**Synthesis of 4.** A solution of the alkylating reagent 3 (1.79 g, 5.75 mmol, 1.35 eq.) in dry MeCN (10 ml) was added dropwise to a well-stirred suspension of K2CO3 (2.94 g, 21.3 mmol, 5 eq.) and tBu3do3a-HBr (2.54 g, 4.26 mmol) in dry MeCN (40 ml) at room temperature. The reaction mixture was stirred at 60 °C for 24 h, filtered, and the filtrate was evaporated in a rotary evaporator. The oily residue was dissolved in CHCl3 (25 ml) and extracted with distilled water (4 × 10 ml). The organic layer was dried over anhydrous Na2SO4 and concentrated in vacuo to yield a yellow oil (3.76 g) containing a crude compound 4 contaminated with an excess of the alkylating reagent 3. The excess alkylating reagent was not removed, and the crude product 4 was used in the next step without purification.

MS-ESI: (+) 577.0 ([M + H]+, calcd 577.3). (–) 574.9 ([M − H]+, calcd 575.3).

**Synthesis of 5.** A portion (3.70 g) of the crude compound 4 obtained above was dissolved in a mixture of CF3CO2H and CHCl3 (1:1, 30 ml). The resulting solution was refluxed for 18 h and evaporated in a rotary evaporator. The oily residue was dissolved in a small amount of distilled water and evaporated (this procedure was then repeated three more times) to produce a yellow oil (3.10 g) containing compound 5, which was used in the next step without purification.

MS-ESI: (+) 565.4 ([M + H]+, calcd 565.4).

**Synthesis of 6.** The crude product 5 (3.00 g) was dissolved in 10% aq. NaOH (50 ml) and stirred for 24 h at 90 °C. Then, the solution was loaded onto a strong anion exchange column (Dowex 1, OH−-form, 1.5 × 20 cm). Impurities were removed by elution with water and the product 6 was eluted with 5% aq. AcOH. Fractions containing the product (as checked by 1H NMR) were combined, filtered and evaporated to give compound 6 (2.21 g) as a brownish oil. The crude product was dissolved in a water:MeOH mixture (1:5, v:v, ∼5 ml) and overlaid with EtOH (∼5 ml) and the mixture was let stand for 2 d. After this period, the solid product was filtered off and dried under vacuum to yield 6 (2.5H2O) (900 mg, 42% based on tBu3do3a) as a white powder.

1H NMR (600 MHz, D2O, pD = 5.88): δ 3.05 (2H, br, CH2CH2NCO); 3.16 (4H, br, (CH2)4NHCNCH3CO); 3.20–3.29 (4H, m, (CH2)3NCH2CH2CO); 3.33–3.44 (12H, br, CH2CH2NCO, CH2CH2NH, (CH2)4NCH2CO); 3.52 (2H, br s, CH2CO); 3.57 (2H, t, JHH = 8.1, CH2NH); 3.70 (4H, br s, CH2CO).

13C{1H} NMR (150.9 MHz, D2O, pD = 5.88): δ 38.69 (1C, CH2CH2NH); 39.45 (1C, CH2CH2NCO); 46.05 (1C, CH2NH); 74.35, 74.50 (4C, CH2NH)}.
49.05 (2C, (CH₂)₂NCH₂CH₂CO); 49.95 (2C, (CH₂)₂NCH₂CO); 50.17 (1C, CH₃CH₂NCO); 51.14 (2C, (CH₂)₂NCH₂CO); 51.79 (2C, (CH₂)₂NCH₂CO); 56.21 (1C, CH₂CO); 57.43 (2C, CH₂CO); 165.09 (1C, CONHN); 173.25 (2C, CH₂CO); 175.78 (1C, CH₂CO).

MS-ESI: (+) 480.9 ([M + Na]⁺, calcd 481.2). (−) 456.8 ([M – H]⁻, calcd 457.3).

Elemental analysis: found (caled for 6.25H₂O, C₁₉H₃₉N₆O₉.5H₂O, Mₚ = 503.6) C: 45.30 (45.32), H: 7.85 (7.81), N: 16.12 (16.69).

Synthesis of H₂do3aNN. Compound 6.25H₂O (415 mg, 0.824 mmol) was dissolved in aq. HCl (10 ml, 1:1) and the resulting solution was stirred for 7 d at 95 °C and evaporated in a rotary evaporator. The oily residue was dissolved in a small amount of distilled water and evaporated to dryness, leaving a glassy solid, which was triturated in dry THF over-night. Next, the product was collected by filtration, and stored leaving a glassy solid, which was triturated in dry THF over-night. A small amount of distilled water and evaporated to dryness, leaving a glassy solid, which was triturated in dry THF over-night. The product was collected by filtration, and stored leaving a glassy solid, which was triturated in dry THF over-night. The result was confirmed by the presence of free Ln(u) ions. The exact concentration of the Ln(u) complexes in the solution was determined using Evans’s method.²²

MS-ESI: (+) 588.9 ([M + Li]⁺, calcd 589.2). (−) 616.7 ([M + Cl]⁻, calcd 617.2).

[Yb(H₂do3aNN)]

MS-ESI: (+) 609.9 ([M + Li]⁺, calcd 610.2). (−) 637.7 ([M + Cl]⁻, calcd 638.2).

PARACEST experiments

All Z-spectra were recorded using a VNMR-S300 spectrometer at 299.9 MHz (B₀ = 7.05 T); 5 mm sample tubes and a coaxial capillary with D₂O and tBuOH as an external standard were used. Solutions of the complexes for PARACEST NMR experiments were prepared in pure water with the pH adjusted using aq. HCl/LiOH solutions and had concentrations in the range of 14–87 mM. Standard pulse sequences for presaturation experiments were used. Saturation offsets were set using the array function (increment 200–250 Hz). Data from the PARACEST experiments were plotted as the dependence of normalized water signal intensity (M₂/M₀) on saturation offset. Here, M₀ represents the magnetization (i.e. intensity) of the water signal without RF saturation and Mₗ corresponds to the water signal when a presaturation pulse is applied. Other experimental parameters are specified in the figure captions.

The magnetization transfer ratio (MTR) was calculated using MTR = M₀−M₀/M₀−M₂ in which M₂=M₀ is the magnetization (i.e. intensity) of the water signal with the use of a presaturation frequency ±Δω away from the bulk water signal.

MRI PARACEST images were measured with a phantom consisting of one vial containing an aqueous solution of buffers [a mixture of 0.025 M 2-(N-morpholino)ethanesulfonic acid (MES) and 0.025 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)] as a standard and nine vials containing solutions of the Eu⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻--; other experimental parameters are specified in the figure captions.

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written in Matlab (Mathworks, Natick, MA, USA). MTR maps were visualized on a false-colour scale in percentage units.

Potentiometry

Potentiometric titrations were carried out in a thermostatted vessel at 25.0 ± 0.1 °C at a constant ionic strength of 0.1 M. The measurements were taken with an HCl excess added to the initial mixture and the mixtures were titrated with a stock NMe$_2$OH solution. An inert atmosphere was maintained by constant passage of argon saturated with water vapour. The ligand concentration in the titration vessel was ~0.004 M.

Ligand protonation constants were determined by standard potentiometric titrations performed in the pH range of 1.6–12.2 (80 points per titration, titrations were carried out four times).

In the cases of the Ln(u)–H$_3$do$_3$aNN systems, the equilibria were established slowly and, therefore, the out-of-cell technique was used in the pH range of 1.6–7.2 (two titrations per system, 25 points per titration). The metal:ligand ratio was 1:1 in all cases. The waiting time was 7 weeks. Then, the potential at each titration point (tube) was determined with a freshly calibrated electrode.

Pre-formed complexes for the determination of their protonation constants were prepared in the following way: in an ampoule, equimolar molar amounts of the ligand and metal stock solutions were mixed and a calculated amount (based on the out-of-cell titration data) of a stock solution of NMe$_2$OH was gradually added to reach pH ~ 7, which corresponds to full complexation according to the out-of-cell titration. Ampoules were flame-sealed and left at 55 °C for 3 d. Aliquots were taken from the final solution, a defined amount of an HCl stock solution was added into these samples and the mixtures were immediately titrated by an NMe$_2$OH stock solution in a way analogous to the procedure described above for the determination of ligand protonation constants in the pH range of 2.3–12.1. The initial volumes were ±5 cm$^3$ for the conventional titrations and ±1 cm$^3$ for the out-cell ones, respectively.

The constants with their standard deviations were calculated by using the OPIUM program package. Overall protonation constants are defined as $\beta_h = [H_hL]/[H^2L]$, and they can be transferred to the consecutive protonation constants log $K_p$ by log $K_p$ = log $\beta_h$ − log $\beta_{h-1}$; it should be noted that log $K_p$ = pK$_A$ of the corresponding protonated species H$_h$L. The overall stability constants $\beta_{h\text{im}}$ are concentration constants defined as $\beta_{h\text{im}} = [H_hL\text{Me}]/[H^2L]\cdot[M^\text{im}]$. The water ion product used in the calculations was pK$_w$ = 13.81. Stability constants of metal hydroxido complexes were taken from the literature. In the text, pH means $-\log[H^+]$. The best fits of experimental data are shown in Fig. S13–S15 and the results are compiled in Tables S1–S3.

Conclusions

The present study revealed significant pH dependence of the Chemical Exchange Saturation Transfer (CEST) effect of selected Ln(u) complexes with the novel macrocyclic ligand H$_3$do$_3$aNN containing a linear diamine pendant arm. The pH dependence is substantial in the pH range relevant for biological systems (pH ≈ 5.5–8.5). Based on these findings, we have shown that the magnetization transfer ratio of CEST signals of the complexes can be used for pH determination by MRI, and it is independent of the concentration of the probes.

Unfortunately, the studied complexes are not fully kinetically inert in acidic solutions and slowly release the free metal ions, which excludes their direct use in medical applications. However, the study brings proof-of-principle of possibility to use a linear diaminic fragment for pH determination using MRI ratiometry.

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References


14 A. E. Martell, R. M. Smith and R. J. Motekaitis, NIST Critically Selected Stability Constants of Metal Complexes, Version 7, Texas A&M University, College Station, TX, 2003.


