Novel polycarboxylated EDTA-type cyclodextrins as ligands for lanthanide binding: study of their luminescence, relaxivity properties of Gd(III) complexes, and PM3 theoretical calculations†

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Novel **EDTA**-type cyclodextrin (CD) derivatives, **AEDTA**, **BEDTA** and **GEDTA**, bearing 6, 7 and 8 bis(carboxymethyl)amino (iminodiacetic acid) groups, respectively, were prepared, and their complexation with Eu(III), Tb(III) and Gd(III) ions was studied. Luminescence titrations and mass spectrometry showed formation of multimetal complexes (**AEDTA** 2 to 3, **BEDTA** mainly 3 and **GEDTA** exactly 4 metal ions), whereas luminescence lifetime measurements revealed the presence of exchangeable water molecules. Semiempirical quantum mechanical calculations, performed by the PM3 method and assessed by DFT calculations on model ligands, indicated efficient multi-metal complexation, in agreement with the experiment. The structures showed coordination of the metal ions in the outer primary side of the CDs *via* 4 carboxylate O atoms, 2 N atoms and a glucopyranose O atom per metal ion. Coordination of water molecules was also predicted, in accordance with experimental results. Calculated bond lengths and angles were in agreement with literature experimental values of lanthanide complexes. Calculated energies showed that complex stability decreases in the order **GEDTA** > **BEDTA** > **AEDTA**. ^{1}H NMR molecular relaxivity measurements for the Gd(III) complexes of **AEDTA**, **BEDTA** or **GEDTA** in water afforded values 4 to 10 times higher than the relaxivity of a commercial contrast agent at 12 MHz, and 6 to 20 times higher at 100 MHz. Solutions of **BEDTA** and **GEDTA** Gd(III) complexes in human blood plasma displayed relaxivity values at 100 MHz 7 and 12 times, respectively, higher than the commercial agent. MTT tests of the Gd(III) complexes using human skin fibroblasts did not show toxicity. Attempts to supramolecularly sensitize the luminescence of the lanthanide complexes using various aromatic CD guests were ineffective, evidently due to large guest–metal distances and inefficient inclusion. The described lanthanide complexes, could be useful as contrast agents in MRI.

Introduction

The cyclodextrins (CDs), cyclic oligomers of α-D-glucose, have become a very important and versatile family of molecular hosts owing to their ability for the inclusion of various molecules, their aqueous solubility and their very low toxicity. CDs are used for drug encapsulation and delivery in pharmaceutical technology[1] but also in analytical separations, and in food and cosmetic technology.[2] Modification of cyclodextrins[3] has afforded derivatives with very interesting properties, including high aqueous solubility,[1] elongated cavities for encapsulation of large guests,[4] enhanced enantioselectivity towards chiral substrates,[5] inclusion of charged guests,[6] for instance nucleotides,[7,8] and novel biological properties such as gene delivery[9–11] and cell membrane penetration.[11] The introduction of a carboxyl group in each α-D-glucopyranose unit provides the CDs with entirely new properties. Direct oxidation of the primary hydroxyls to carboxyl groups has been reported,[12,13] yielding hosts with a propensity to dimerise. Reaction of carboxyalkylthiols with per(6-bromo-6-deoxy)CDs has afforded carboxylated hosts that bind to a positively charged steroidal muscle relaxant very efficiently.[4] Per(6-carboxyalkylthio-6-deoxy)-β-CD derivatives have been used to bind cationic guests[14] and analogous γ-CD derivatives to decelerate hydrolysis of penicillin drugs.[15] Methylated per(6-carboxymethyl)-β-CDs have been shown to form a ternary complex with cytochrome c and porphyrins.[16] In general, carboxylated CDs are also expected to display excellent binding properties with lanthanide cations: it is well known that the latter behave as typical hard acids and interact preferentially with hard bases, such as carboxylates, to form strong complexes.[17] The high charge of lanthanide(III) cations[17,18] favors complexation by polyanionic ligands owing to a large electrostatic contribution to the binding. Thus, an ideal candidate for binding lanthanide ions would be a CD having carboxyl groups connected to its core *via* suitable linkers.

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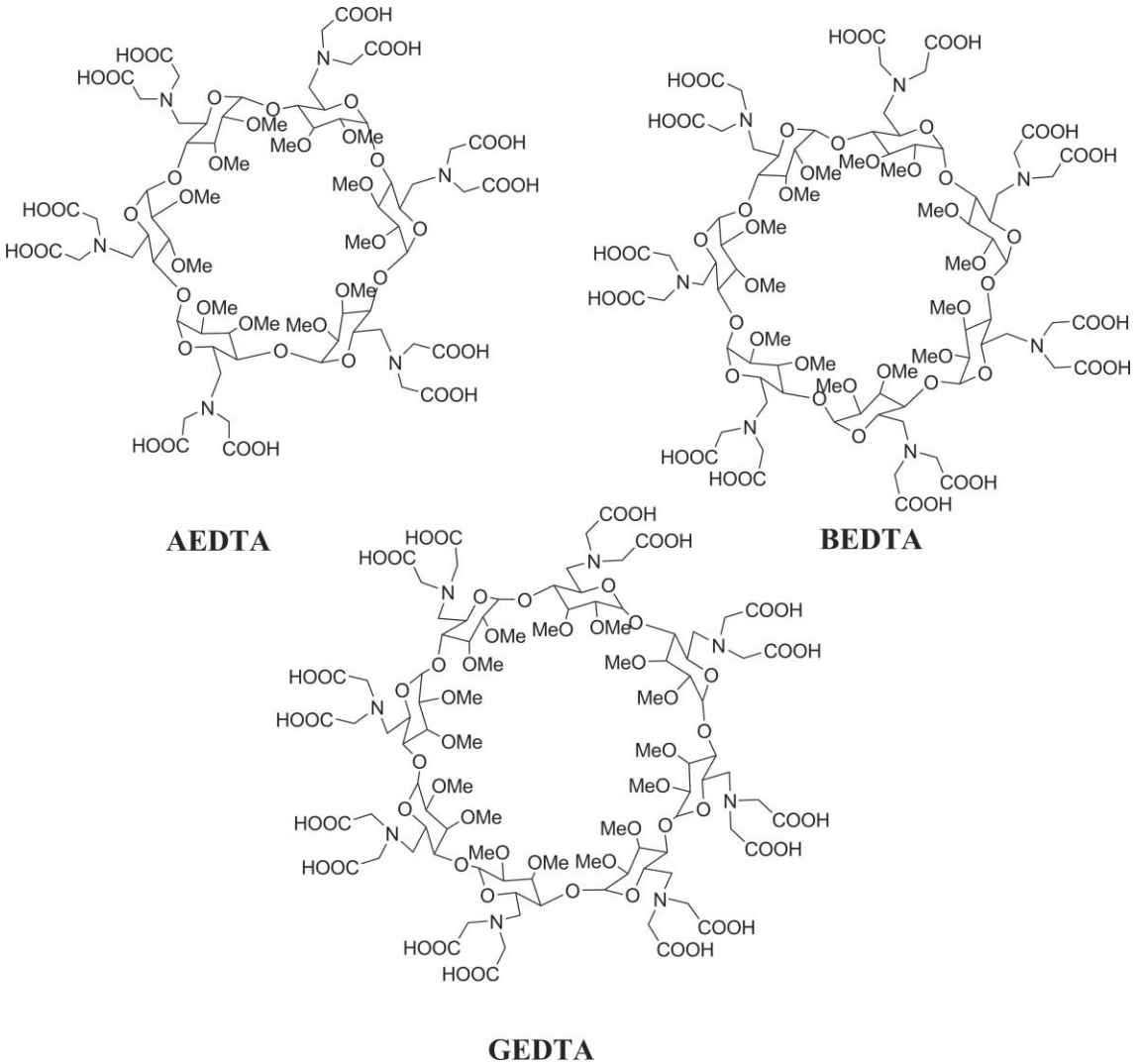


Scheme 1 Novel per[6-bis(carboxymethyl)amino-6-deoxy-2,3-methyl]-α-, -β- and -γ-CDs, **AEDTA**, **BEDTA** and **GEDTA**.

Literature examples include lanthanide complexes [Dy(III) or Yb(III)] with carboxymethyl-CDs that act as chiral shift reagents in NMR spectra of aromatic cationic guests.[19] Also, a 3 : 1 fluorescent monocarboxylated-βCD/Tb(III) complex was found to include bile salts in the form of higher aggregates. A lanthanide complex with one EDTA-like chain linking the wider (secondary) sides of two βCDs has been shown to form with aromatic guests a metallo-supramolecular luminescence sensitization system.[20] Mono-[bis(carboxylatomethyl)amino]cyclodextrin[21] derivatives and their Eu(III) complexes have been screened as enantioselective hosts.[21] Polycarboxylate dendrons grown on the primary side of β-CD have also been synthesized and shown to retain their inclusion complexation properties.[22]

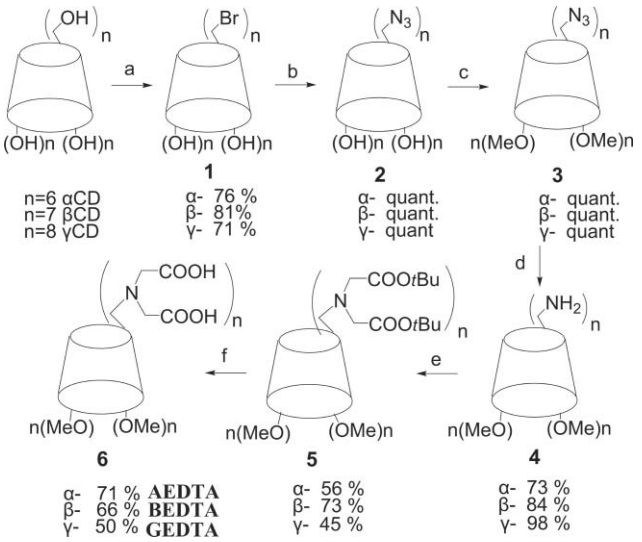
Introduction of bis(carboxymethyl)amino (iminodiacetic acid) groups on all primary hydroxyl sites of CDs has been a target in our group in order to investigate the ability of the resulting EDTA-type CDs to coordinate with lanthanide ions and study their foreseen properties. The successful preparation of per[6-bis(carboxymethyl)amino-6-deoxy-2,3-methyl]-α-, -β- and -γ-cyclodextrin (**AEDTA**, **BEDTA** and **GEDTA**, respectively, Scheme 1) is being reported here. Their complexes with lanthanide ions are studied and structures are examined by semiempirical calculations at the PM3 level. Interestingly, ^{1}H NMR T_1 measurements of the Gd(III) complexes of the novel EDTA-type CDs in water gave very high relaxivity values, especially at 100 MHz. These properties, along with the presence of fast exchanging water molecules and of multiple Gd(III) ions per complex, are attractive features for applications of the complexes in MRI spectroscopy.

Results and discussion

Synthesis of EDTA-type CDs

The synthetic steps towards the polycarboxyl cyclodextrins are outlined in Scheme 2. Products **1**, **2**[23] and **3**[24,25] were prepared in high purity and good yields according to literature procedures. Methylation of the secondary hydroxyl groups of macrocycle **3**[25] was necessary in order for the corresponding amino derivatives **4**[25] to react efficiently with excess *t*-butyl bromoacetate to give, after column chromatography, pure products **5**.

2D-NMR spectroscopic analyses verified the completeness of substitution at the methylene carbon atoms of the core CDs



Scheme 2 Conditions: a.[23] DMF, 70 °C, PPh_3, Br_2, O=PPh_3 content in the products < 1%; b. DMF, 60 °C, NaN_3; c. THF, 25 °C, NaH, MeI; d. DMF, 25 °C, NH_3(aq), PPh_3; e. CH_3CN, reflux, *t*-butyl bromoacetate, KI, K_2CO_3; f. $CHCl_3$, 25 °C, TFA.

in products **5** and the presence of two *t*-butoxycarbonylmethyl arms per nitrogen atom. The ^{1}H (ESI, Fig. S1a†) and ^{13}C NMR spectra (Fig. 1a) showed only one signal per group of hydrogen or carbon atoms, thus revealing a high overall molecular symmetry. Notably, a single carbonyl group was observed for each of the products **5**. Similarly, MALDI-TOF mass spectra gave the expected molecular ion as the base peak in each case (ESI, Fig. S2†). The novel polycarboxylated CDs, **AEDTA**, **BEDTA** and **GEDTA** were obtained from compounds **5** by trifluoroacetic acid hydrolysis.

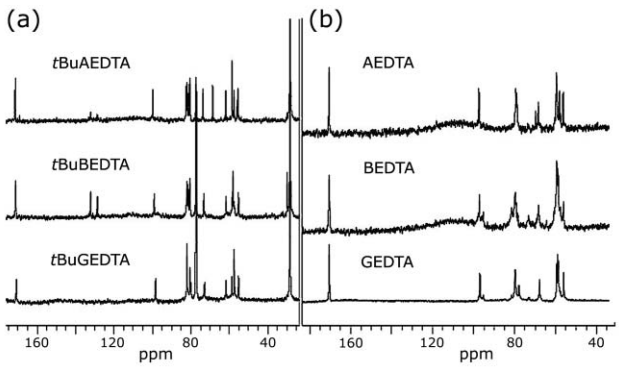


Fig. 1 ^{13}C NMR spectra (D_2O, 125 MHz, 298 K) of: (a) *t*-butyl esters **5** and (b) EDTA-CDs **6**. The two signals near 130 ppm in (a) are due to O=PPh_3 impurities, absent in the spectra in (b).

The ^{1}H NMR spectra of compounds **5** (ESI, Fig. S1a†) and **6** (ESI, Fig. S1b†) are different in that the sharp peaks of the *t*-butyl derivatives **5** changed to rather broad peaks in the carboxyl derivatives **6**. Compared to the spectra of compounds **5** (Fig. 1a), the ^{13}C NMR spectra of **6** (Fig. 1b) show rather broad and more than one signal per type of carbon atom. However, both sets of spectra display single or nearly single, sharp and strong carbonyl carbon peaks near 170 ppm (Fig. 1a,b). The observed broadness in the NMR spectra could be attributed to the presence of methoxy groups at the secondary side of the CDs, because methylation increases the macrocyclic flexibility[26] by disrupting the H-bonding network of the parent CD.[27] Another more probable source of multiple signals is the anticipated variable degrees of protonation. The integrity of **AEDTA**, **BEDTA** and **GEDTA** was additionally confirmed by the MALDI-TOF spectra that gave the exact expected masses both in the positive and negative polarity modes (ESI, Figs S3–S5†), and by analytical data.

Use of per(6-amino-6-deoxy)CDs (with hydroxyl groups at the wider side), instead of the methylated analogues **4**, in the reaction with *t*-butyl bromoacetate, afforded only mixtures. Attempts to introduce two carboxymethylamino moieties per glucopyranose unit using per(6-bromo-6-deoxy)-β-CD (**1**) and iminodiacetic acid gave unsatisfactory results, despite various reaction conditions. In another approach towards EDTA-type CDs, per(6-iodo-6-deoxy)-β-CD reacted successfully with 2-(*t*-Boc-amino)ethanethiol to afford per(*t*-Boc-aminoethylthio-6-deoxy)-β-CD, the amino group was freed of its protection by trifluoroacetic acid and the resulting pure per(6-aminoethylthio-6-deoxy)-β-CD[28] was subjected to reaction with chloroacetic acid in order to directly introduce two carboxymethylamino groups on each nitrogen atom. The solid recovered from that attempt had unexpectedly low aqueous solubility at various pH values and did not give satisfactory analytical data; therefore this approach was abandoned.

Complexes of lanthanide(III) ions with EDTA-type CDs

The complexes with lanthanide(III) ions were prepared by addition of lanthanide salts to a solution of the given carboxylated CD ligand in pure water. Although the large paramagnetism of Tb(III) and Gd(III) prohibits analysis of the complexes by NMR, it is possible to obtain NMR spectra of the Eu(III) complexes. The ^{1}H NMR spectrum (Fig. 2a) showed significant broadening of the signals and large shifts ranging from +14.4 to −7.6 ppm, typical of europium complexes[29] signifying complexation of Eu(III) with the ligands. Further evidence for lanthanide ion binding on the carboxyl groups was obtained from the IR spectra (Fig. 2b). Thus, comparison of the IR spectra of the CD ligands on their own and in the presence of Tb(III) or Eu(III) show that the C=O stretching band of **BEDTA** undergoes a considerable shift of 50 cm^{-1} due to the formation of the complex with Eu(III).

The stoichiometry of the complexes was determined by titrating each metal ion solution with an aqueous buffered solution of the CD ligand at pH 7 and recording the luminescence emission spectra. During titration, increased lanthanide emission is observed because the CD ligand provides efficient isolation of the metal ion from the surrounding O–H oscillators of the solvent, responsible for the quenching of the lanthanide emission, and also because displacement of water molecules from the first coordination sphere of the Eu(III) ion occurs upon complexation.[20] The area of the most sensitive transition for Tb(III) (λ_{em} = 545, $^5D_4 \rightarrow {}^7F_5$) and Eu(III) ($\lambda_{em}$ = 617, $^5D_0 \rightarrow {}^7F_2$) was plotted as a function of the equivalents of CD ligand added and the plots are shown in Fig. 3. Since variations in the chemical properties of lanthanides are barely discernible, similar binding behavior and titration curves upon coordination of each metal with the same CD ligand are expected. Titration of Eu(III) solution with **AEDTA** (Fig. 3b) gives a very slowly growing binding curve, a shallow break-point and a rising

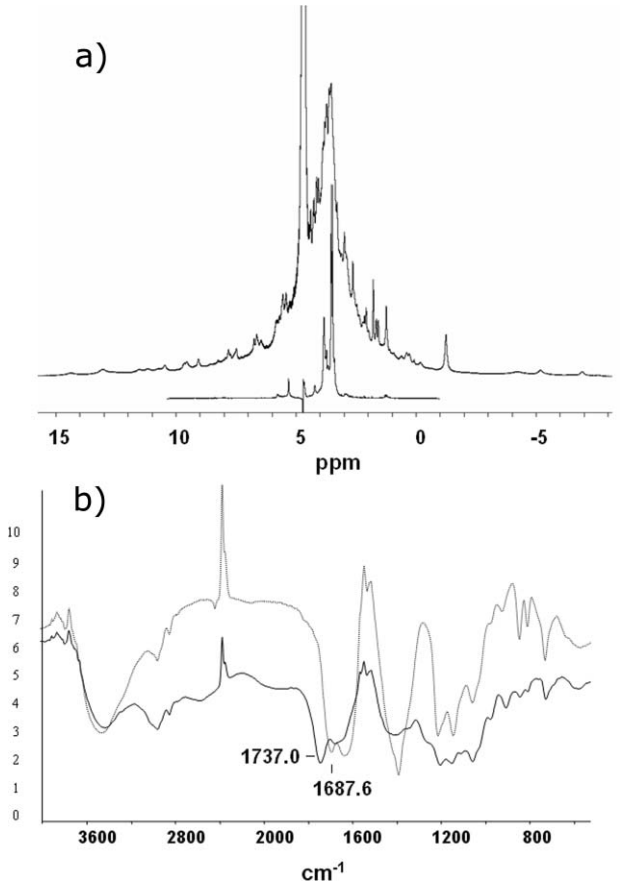


Fig. 2 (a) ^{1}H NMR spectrum of the **BEDTA** (bottom, residual water signal at 4.75 ppm suppressed) and **BEDTA**-Eu(III) complex (top) in D_2O (500 MHz, 298 K). (b) IR spectrum of (**BEDTA** (—) and its Eu(III) complex (···).

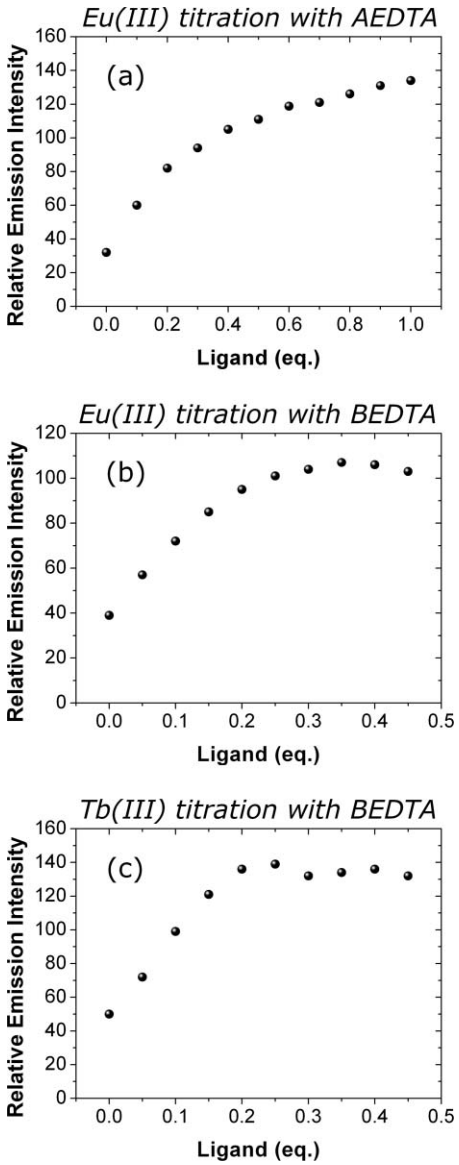


Fig. 3 Titration plots of $EuCl_3$ and $TbCl_3$ solutions in buffered H_2O (HEPES 10.0 mM) by addition of (a) **AEDTA** and (b, c) **BEDTA**.

plateau. This shape indicates that more than one species is formed with a stoichiometry up to 1 : 3 [**AEDTA** : 3Eu(III)], whereas co-existence of oligomeric species is possible, as well. ESI-TOF mass data of this complex recorded in negative ion mode showed peaks corresponding to complexation of one **AEDTA** with two Eu(III) ions ([**AEDTA** + 2Eu(III) − 58(CH_2COOH) − 9H^+]$^{3-}$, m/z = 689.1, 95%, corresponds to mass 2070), and three Eu(III) ions ([**AEDTA** + 3Eu(III) − 11H^+]$^{2-}$, m/z = 1138.2, 60%, corresponds to mass 2278). The mass spectrum overall appeared as a mixture of species (ESI, Fig. S6†). The Eu(III) and Tb(III) titration curves with **BEDTA** (Fig. 3b,c) were very similar to each other with clear break-points at ~0.3 eq. and unambiguous plateaus. Formation of a stoichiometric complex in each case, [**BEDTA**·3Eu(III)] is thus inferred. Examination of the corresponding low-noise ESI-TOF mass spectrum (ESI, Fig. S6†) revealed strong peaks arising singly from the [**BEDTA**·3Eu(III) − 12H^+]$^{2-}$ ion, (m/z = 1290.72, base peak, corresponds to mass 2583) and [**BEDTA**·3Eu(III) − 11H^+]$^{3-}$ ion (m/z = 860.14, 50%, corresponds to mass 2583). In addition a very low intensity peak, assigned as the [2**BEDTA**·6Eu(III) − 3H^+]$^{3-}$ species (m/z = 860.14, 7%) was also observed. Peaks arising from [**BETDA**·4Eu(III)]$^{x-}$ (x = 2 or 3) were not found. The above show that stoichiometry results from titration plots and from ESI-TOF mass data for the lanthanide complexes are in very good agreement. The corresponding ESI-TOF mass spectra of **GEDTA** complex with Gd(III), recorded in positive ion mode (ESI, Fig. S6†) revealed a strong peak arising from the hydrated complex [**GEDTA** − 12H^+ + 4Gd^{3+} + 2Na + H + H_2O]$^{3+}$ ion (m/z = 1041.81 in non-smoothed spectrum, base peak), along with a peak arising from the [**GEDTA** − 12H^+ + 4Gd^{3+} + 2Na + H_2O]$^{2+}$ ion (m/z = 1562.7). Signals arising from complexes coordinated with less than four Eu(III) ions were not observed.

In summary, **AEDTA** forms mixtures of complexes with two and three lanthanide ions, **BEDTA** coordinates largely with three lanthanide ions and **GEDTA** complexes exclusively with four lanthanide ions. All metal complexes are completely soluble in water.

The hydration number of the complexes can be calculated by measuring the lifetime of the excited ions in H_2O, where the bound water molecules quench the lanthanide luminescence, and in D_2O where the quenching is very inefficient. Horrocks and Sudnik[30–32] have shown that the number of inner sphere coordinated water molecules, q, with an estimated uncertainty of ±0.5, is given by

Table 1 Luminescence lifetimes (τ) in H_2O and D_2O of the complexes and number of water molecules coordinated (q)

Complex	τ_{H_2O}/ms	τ_{D_2O}/ms	q (Eqn 1)	q (Eqn 2)
BEDTA- Tb(III)	1.25	2.22	1.47 ± 0.5	1.45 ± 0.3
BEDTA-Eu(III)	0.56	2.15	1.39 ± 0.5	1.29 ± 0.3

eqn (1), where τ_{H_2O} and τ_{D_2O} are the experimental excited-state lifetimes (in ms) in H_2O and D_2O solutions, respectively. A is an empirical parameter that equals 1.05 for Eu(III) and 4.2 for Tb(III). This expression was modified later to account for the smaller effect of N–H and C–H oscillators and of closely diffusing but non-coordinated water molecules,[33] resulting in eqn (2), which provides the hydration number with an estimated accuracy of 20%:

$$q = A\,(1/\tau_{H_2O} - 1/\tau_{D_2O}) \quad (1)$$

$$q^{Eu} = 1.2[(1/\tau_{H_2O} - 1/\tau_{D_2O}) - 0.25],$$
$$q^{Tb} = 5[(1/\tau_{H_2O} - 1/\tau_{D_2O}) - 0.06] \quad (2)$$

Lifetime measurements were carried out in H_2O and D_2O, following direct excitation of the lanthanide ion [Eu(III): λ = 394 nm; Tb(III): λ = 368 nm]. In each case mono-exponential decay curves were observed and the data obtained allowed an estimation of the number of water molecules totally coordinated (hydration states) (Table 1). The numbers indicate that on average 1.5 water molecules are coordinated with each **BEDTA**-lanthanide complex. The above method involves measurements with Eu(III) and Tb(III) ions that are positioned on either side of Gd(III) in the periodic table and have similar radii; therefore, the results in Table 1 are a very good indication of the hydration number in the corresponding Gd(III) complexes. The above experiments proved the presence of exchangeable water molecules in lanthanide complexes with the CD-ligands, a very important property for the utilization of the respective Gd(III) complexes as contrast agents for magnetic resonance imaging. The presence of the metal coordinated water molecules was also predicted theoretically for all EDTA-CDs, as will be shown in the calculations section.

Relaxivity studies

AEDTA, **BEDTA** and **GEDTA** were shown above to form water soluble multimetal complexes with lanthanide ions and to possess exchangeable water molecules in their coordination sphere, whose rapid exchange with the surrounding water molecules in aqueous solution would affect the ^{1}H NMR relaxation times T_1 (and/or T_2) of all the solvent protons. The relaxivity, r (mM^{-1} s^{-1}) defined[34] as the paramagnetic relaxation rate enhancement of the water proton resonance referred to 1 mM concentration of the agent, such as a Gd(III) complex in aqueous solution, is directly proportional to the number of bound water molecules. In addition, the longitudinal proton relaxation rate, R_1 ($\equiv 1/T_1$), varies with the distance, d, between the Gd(III) ion and the water proton in a d^{-6} relationship.[33] Coordination of Gd(III) ions to high molecular weight molecules results in increased rotational correlation time, τ_R, which contributes to the effective correlation time, τ_C, that modulates the dipole–dipole relaxation process thus increasing relaxivity.[34] Finally, the presence of many Gd(III) ions per ligand should enhance the molecular relaxivity even more. In light of the above, Gd(III) coordination with the present monodisperse, polycarboxylated EDTA-type CDs, is anticipated to result in increased relaxivity values of their resulting complexes in aqueous solution.

AEDTA, **BEDTA** and **GEDTA**, each in stoichiometric excess, reacted with $Gd(NO_3)_3$, and the relaxivities of the resulting solutions were measured at 0.285 Tesla (12.2 MHz) and at 2.35 Tesla (100 MHz). The T_1 relaxation times, of the order of ms, of each solution were measured at five different Gd(III) concentrations and the relaxivity, r, was calculated as the slope of the linearly fitted curve according to eqn (3),[34]

$$(1/T_1)_{obs} = (1/T_1)_d + (1/T_1)_p = (1/T_1)_d + r[\mathrm{Gd(III)}], \quad (3)$$

where the subscripts d and p refer to diamagnetic and paramagnetic contributions to the overall relaxation. The results obtained are shown in Table 2.

In the solutions of the CDs without Gd(III) ions and in the blood plasma, the relaxation times of the water signal were of the order of s (*e.g.* T_1 of **GEDTA** solution was 2 s, thus the contribution of the diamagnetic term was negligible). At both 12 MHz and 100 MHz the molecular relaxivity increases from **AEDTA** to **GEDTA**, as anticipated due to both increased molecular weight of the complexes and of the number of coordinated Gd(III) ions. The relaxivities per Gd(III) ion also increase from **AEDTA** to **GEDTA**, and actually they are significantly greater than the relaxivities of commercial agents such as the magnevist® (gadopentate dimeglumine, DTPA) and dotarem® (gadoterate meglumine, DOTA), both ~4 mM^{-1} s^{-1} at 20 MHz.[34] In a control experiment, the relaxivity of $Gd(NO_3)_3$ at 12 MHz was measured and found to be alarmingly similar to that of the complex with **AEDTA** at the same concentration. However, at 100 MHz the value decreased, as expected for $Gd(NO_3)_3$, whereas the relaxivity of the **AEDTA** complex increased significantly, thus proving that the observed high relaxivity values genuinely arise from efficient complexation of Gd(III) with the title CDs. Finally, relaxivity measurements of

Table 2 Relaxivities (mM^{-1} s^{-1}) of Gd(III) complexes with EDTA-CDs at 25 °C

	12 MHz in water			100 MHz in water			100 MHz in blood plasma
Compound	R^2 [a]	Molecular relaxivity	Relaxivity/ Gd(III) ion	R^2 [a]	Molecular relaxivity	Relaxivity/ Gd(III) ion	Molecular relaxivity
$Gd(NO_3)_3$	0.9988	16.5 ± 0.56		0.9964	11.2 ± 1.51		
AEDTA	0.9922	16.4 ± 1.19	6.50[b]	0.9891	23.2 ± 1.99	9.30[b]	
BEDTA	0.9985	26.5 ± 0.83	8.80	0.9971	31.4 ± 1.34	10.5	28.0 ± 2.51[c]
GEDTA	0.9908	41.0 ± 3.23	10.2	0.9905	92.9 ± 9.33	23.2	50.2 ± 0.90[d]

[a] Goodness of fit to the linear relationship (3); [b] calculated using 2.5 Gd(III) ions per **AEDTA**. [c] R^2 = 0.9920; [d] R^2 = 0.9995

the complexes of **BEDTA** and **GEDTA** at 100 MHz in human blood plasma were additionally carried out in order to evaluate the stability of the complexes under physiological conditions over ~2.5 h. **BEDTA** complex displayed a ~10% reduction of its relaxivity value, approaching that in water at 12 MHz. **GEDTA** complex suffered a 45% reduction; however, the relaxivity in blood plasma remained exceptionally high and ~25% higher than in water at 12 MHz. The results show that the stability of the complexes is sufficient to sustain high relaxivity values in challenging media.

Relaxation theory as outlined by the Solomon–Bloembergen–Morgan equations,[34,35] which describe relaxation as a function of the magnetic field, predicts a change in the relation between relaxivity and rotational correlation time at high fields (more than 60 MHz), *i.e.* when τ_R is increased (*e.g.* from 0.1 to 1 ns) relaxivity increases significantly.[34] Accordingly, our molecules with a relatively long τ_R display elevated relaxivities at 100 MHz: the Gd(III)-**GEDTA** complex at 100 MHz has an *r* value more than double the value at 12 MHz. This trend is opposite to that observed upon examining the NMRD curves of small clinically approved Gd(III) chelates, such as DOTA and DTPA, whose relaxivities at 100 MHz are smaller than at 10 MHz.[34] The presently observed direct relationship between relaxivity and rotational correlation time is an important feature since magnetic imaging applications use increasingly higher magnetic fields. Similar examples from the literature include a moderate molecular weight starburst-shaped heterometallic chelate with six Gd(III) ions,[35] which displayed its highest relaxivity at low temperature (5 °C) and at ~100 MHz, glycoconjugates of Gd(III),[36] and β-cyclodextrin heptasubstistuted with Gd(III)-DTPA complexes.[37] Previous studies have also shown that poly-β-CD complexes with Gd(III) chelates,[38] or multiple complexes of β-CD with Gd(III) chelates with large hydrophobic parts,[39] or even covalently bound Gd(III)-DTPA-βCD-polypeptide conjugates[40] display increased relaxivities per Gd(III) ion at a given magnetic field, compared with Gd(III) complexes of small organic ligands, due to increased molecular weight of the resulting system.

Cell toxicity studies

The complexes of **AEDTA**, **BEDTA** and **GEDTA** with Gd(III), along with the widely used commercial MRI contrast agent magnevist® were tested for possible cytotoxic activity on normal human skin fibroblasts. As shown in Fig. 4, there was no statistically significant reduction of the cells' viability at the concentration-range tested after 72 h of incubation in the presence of the complexes. Doxorubicin hydrochloride, used as a positive control in these experiments, was cytotoxic, as expected, with an IC_{50} value of 1.38 (±0.49) μM (not shown). The highest concentration of magnevist® tested (*i.e.* 1 mM) has been reported to be cytotoxic for a human breast cancer cell line[41] and to a lesser extent for the murine fibroblast cell line NIH/3T3.[42] The fact that 1 mM magnevist® is not cytotoxic for normal human cells is probably due to their lower proliferation rate and/or to a lower uptake rate compared to the above-mentioned immortalized cell lines. In the present case evaluation of cellular uptake of the Gd(III)-CD complexes was not feasible; however, no uptake could mean that the complexes can act as extracellular agents, like several other MRI agents currently available.[34]

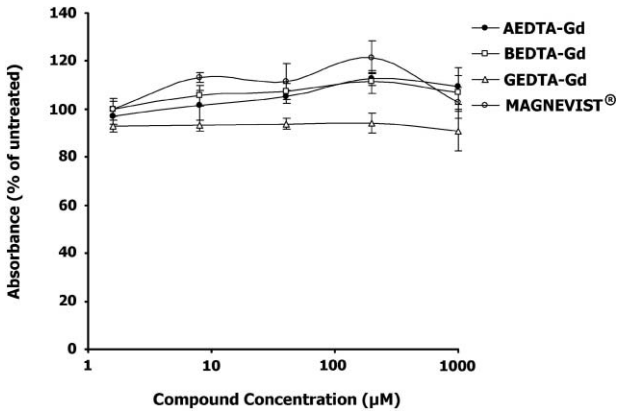


Fig. 4 Viability of human skin fibroblasts exposed to the test compounds for 72 h as expressed by the % absorbance estimated by the MTT-method. Each point is the average of four replicates (error-bars indicate standard deviation).

Attempted sensitized lanthanide emission

Eu(III) and Tb(III) complexes usually display bright red and green luminescence, respectively, which originates from transitions between their ^5D_n excited states to the ^7F_J ground states. The inclusion of aromatic guests in the CD cavity of the lanthanide-CD complex would be a possible indirect method to sensitize the emission of the lanthanides, *i.e.* to irradiate the guest and obtain efficient energy transfer to the metal ion resulting in its augmented emission. Several potential guests suitable to enter the CD cavity (*t*-butyl benzoate, naphthalene, acetophenone, *p*-aminoacetophenone, benzene, benzophenone) were screened with **BEDTA**, in aqueous solutions, as well as in deoxygenated aqueous solutions. No energy transfer was detected in the case of Eu(III), whereas in the case of Tb(III) complexes inefficient energy transfer was detected with naphthalene only. This could be attributed to either large distance between the metal center and the guest (as shown below by the theoretically calculated structures), or to low binding constants of the above guests with the specific CDs, both properties being a consequence of flexible macrocyclic structure. Similar behaviour (weak to very low sensitization) has been shown in several cases in the literature.[43,44]

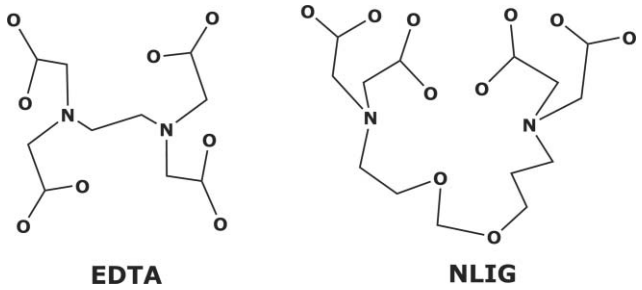
Theoretical calculations

Quantum mechanical calculations were performed in order to determine the structures of the complexes between **AEDTA**, **BEDTA** and **GEDTA** with Eu^{+3}, Gd^{+3} and Tb^{+3} ions, their stability, and their capacity to coordinate water molecules. The reliability of the theoretical results obtained by the semiemprical PM3 method was assessed by benchmark density functional (DFT) calculations in complexes of the above lanthanide ions as well as La^{+3} and Lu^{+3} with smaller fully deprotonated ligands, designed as models of the metal binding sites of the substituted CDs, $(HOCOCH_2)_2NCH_2CH_2N(CH_2COOH)_2$ (**EDTA**) and $(HOCOCH_2)_2NCH_2CH_2OCH_2O(CH_2)_3N(CH_2COOH)_2$ (**NLIG**) (Scheme 3).

The formation of lanthanide coordination complexes may be considered as a primarily electrostatic interaction between the tri-positive lanthanide ion and the negatively charged $-N(CH_2COO^-)_2$

Table 3 Geometrical parameters of EDTA-CDs calculated by PM3

Compd.	***d*/Å**	rms ***D*/Å**	**Tilt angles** (°)
Neutral CDs			
AEDTA	3.97–4.49	0.29	4.4–45.1
BEDTA	4.20–4.54	0.45	5.9–33.3
GEDTA	4.14–4.55	0.96	21.4–75.7
CD Anions			
AEDTA	4.37–4. 46	0.05	4.4–18.6
BEDTA	4.56–4.72	0.20	5.1–26.0
GEDTA	4.55–4.97	0.49	7.9–69.4
CD/Gd(III) complexes			
AEDTA	3.56–4.56	0.36	6.2–34.2
BEDTA	3.64–5.10	0.40	10.2–54.0
GEDTA	3.85–4.78	0.52	13.6–56.6



Scheme 3 The ligands employed as models of the EDTA-CDs metal binding sites.

groups appropriately arranged in order to coordinate with the metal center.

The PM3 computed binding energies for the complexes of the lanthanides La(III), Eu(III), Gd(III), Tb(III) and Lu(III) with model ligands, markedly (by *ca.* 1500 kJ mol^{-1}) lower than those calculated by DFT, indicate that the semiempirical PM3 energies are less accurate in an absolute sense (ESI, Table S1†). However, PM3 is able to reproduce up to Gd the trend predicted by DFT *i.e.* that complex stability increases with the size of the lanthanide atom.

CD structures. The PM3 optimized structures of the three CDs in their neutral as well as in their anionic forms are shown in Fig. 5.

In the neutral forms the substituents, extending over the primary side of the CD torus, contribute to the elongation of the cavities. In **AEDTA** and **GEDTA** three substituents cluster together by weak H-bonds between their carboxyl groups and obstruct the primary entrance of the cavities. All glucose units have the usual $^4C_1$ chair conformations.[27] There is extensive distortion of the EDTA-CD macrocycles (less in **BEDTA**, most in **GEDTA**), as reflected by their geometrical parameters (Table 3). The distances (**d**) between the oxygen atoms bridging the glucose units [O4n · · · O4(n-1)] vary considerably. The same is true for the root mean square distances (**D**) of the O4n atoms from their mean plane.

The tilt angles that show the degree of tilting of glucose residues with respect to the above plane also vary widely. In **AEDTA** all glucose units tilt with their primary side towards the cavity, whereas one unit in **BEDTA** and three in **GEDTA** tilt with the secondary side inwards. High tilting is not unusual in the per-substituted CDs: high tilt angles as in **GEDTA** have been observed in the crystal structure of an acetylated CD.[45] Upon full deprotonation (CD anions), all macrocycles become more

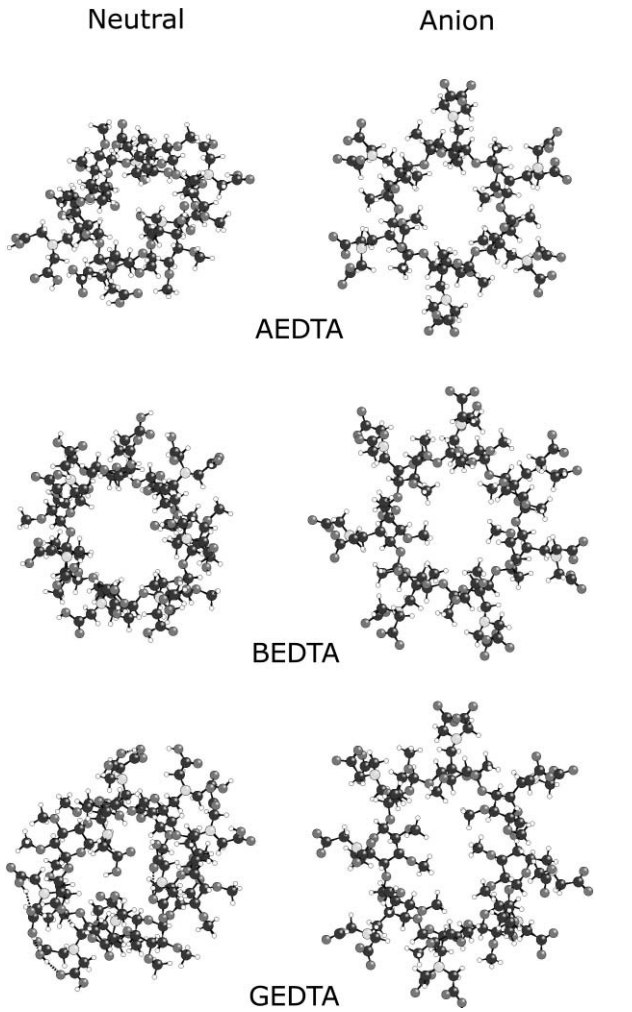


Fig. 5 Molecular models of the CDs in their protonated (neutral) and fully unprotonated (anionic) forms drawn to scale. C: dark grey; O: medium grey; N: light grey, H: white.

symmetrical compared to the neutral forms, all substituents on the primary side rotate outwards as a result of mutual repulsion of the charged carboxyl groups and the glucose units are stabilised in the skew-boat (twist) conformation (Fig. 5). The macrocycles become more symmetrical compared to the neutral forms. Note that distortion of the present EDTA-CDs is possible, due to the absence of the H-bonds between O-3*n* and O-2(*n*–1) atoms[46] that keep the macrocycles of natural CDs rigid,[27] a consequence of methylation of the secondary hydroxyl groups.

Structures of lanthanide complexes. The successive uptake of the lanthanide cations Eu^{+3}, Gd^{+3} and Tb^{+3} by the fully deprotonated CDs leads to a series of complexes whose total PM3 calculated binding energies are listed in the ESI, Table S2.† The corresponding structures show coordination of the metal ions in the outer primary side of the CDs *via* 4 carboxylate O and 2 N atoms of two adjacent substituents. In addition, an oxygen atom of the nearest glucopyranose ring appears to be coordinated with the metal atom in all cases with an average $Ln–O_{(glucopyranose\ ring)}$ bond length of *ca.* 2.5 Å. Thus, vacant coordination sites on the generally 9-coordinated lanthanide cations are available for water molecules. In general, the stability of the complexes increases with the size of

Table 4 Average values for the distances (in Å) between lanthanide atoms and coordinated heteroatoms (carboxy O, N) for model ligands, calculated by PM3 and DFT and for the complexes of substituted CDs calculated by PM3

	CDs	Model Ligands	
Bond[a]	PM3	PM3	DFT
La–O		2.512	2.378
La–N		2.680	2.710
Eu–O	2.448	2.410	2.275
Eu–N	2.607	2.600	2.610
Gd–O	2.398	2.363	2.257
Gd–N	2.578	2.565	2.595
Tb–O	2.409	2.378	2.247
Tb–N	2.599	2.585	2.525
Lu–O		2.312	2.153
Lu–N		2.480	2.395

[a] $Ln–O_{(glucopyranose\ ring)}$ *ca.* 2.5 Å

the CD core, leading to stronger lanthanide complexes in the order **GEDTA** > **BEDTA** > **AEDTA.** The PM3 energies suggest that the maximum number of lanthanide atoms accommodated into each substituted CD is 3 for **AEDTA** and 4 for **GEDTA**, whereas a value of 3 is more likely for **BEDTA** (Fig. 6), in agreement with the experimental findings. The accuracy of the distances between the metal and coordinated heteroatoms calculated by the PM3 method was assessed by a comparison with the average values calculated by DFT for the model ligands (Table 4).

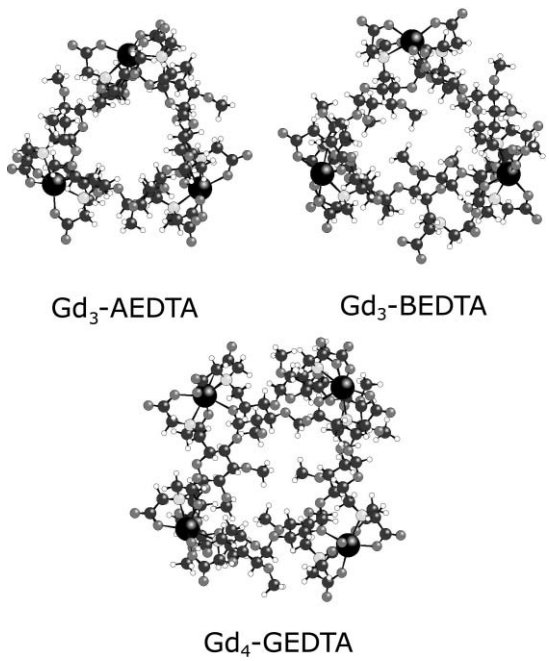


Fig. 6 Molecular models of Gd_3-AEDTA, Gd_3-BEDTA and Gd_4-GEDTA complexes, drawn to scale. Color code as in Fig. 5; Gd atoms are depicted in black.

The DFT values display a reduction of the bond lengths along the lanthanide series, as expected on the basis of the lanthanide contraction. A similar trend is observed for the PM3 results.

The best agreement between PM3 and DFT is observed in the Ln–N followed by Ln–O bond lengths. The PM3 method overestimates Ln–O distances by more than 0.1 Å; however, the computed values fall within the range of those obtained by X-ray crystallography.[34] The detailed list of the calculated bond lengths is shown in the ESI, Table S3.†

For the model ligand complexes, the average values of the C–H, C–C, C–N and C–O bond lengths at the PM3 level of theory are calculated to be 1.101, 1.527, 1.540 and 1.301 Å, respectively, with a maximum unsigned deviation of only 0.06 Å from the DFT values.

Although the iminodiacetate substituents in the anionic CDs are fully extended outwards, they fold back to form dative bonds with the lanthanide ions. In doing so, the macrocycles loose their circular cross-section and the glucopyranose units assume various conformations: in **Gd_3-AEDTA** all units tilt with the primary side inwards, two acquire the boat and four the chair conformation. In **Gd_3-BEDTA**, three units tilt outwards (two bound to Gd(III) and one carrying the free substituent), five units acquire the chair conformations, one remains in skew-boat and one assumes the boat conformation. In **Gd_4-GEDTA**, half of the units in diametrically opposite positions tilt outwards. The overall structure is more symmetric. The outward tilting of glucopyranose pairs keeps the Gd(III) ions completely out of the cavity, whereas in the two inward tilting pairs the Gd(III) ion is located almost above the side walls of the macrocycle. The outward tilting of the three and four glucopyranose rings in **BEDTA** and **GEDTA**, respectively, brings the corresponding OMe groups on C-3 positions inwards, thus blocking the secondary entrance of the cavities. In light of the above, **BEDTA** and **GEDTA**, being large and flexible macrocycles, seem to have their cavities obstructed, and thus the inclusion of guest molecules may be limited, whereas any guest entering the cavity of **AEDTA** from the secondary side, which is not obstructed, would be far from the metals, thus sensitized energy transfer from guest to metal would not be efficient. The above justify the experimentally observed lack of supramolecular sensitization of the lanthanide luminescence.

Coordination of water molecules. DFT and PM3 calculations suggest that the lanthanide complexes of model ligands and EDTA-CDs may be associated with one or two water molecules to yield hydrates in order to fulfill a coordination number of 9 around the metal atom. In particular for the EDTA-CDs complexes, average $Ln–O_{(water)}$ distances are 2.470, 2.430 and 2.412 Å for Eu(III), Gd(III) and Tb(III), respectively. These are very similar to the distances found in water complexes of the model compounds and compare well with the existing experimental values from X-ray crystallography.[34]

Conclusions

Novel EDTA-type CDs (**AEDTA**, **BEDTA** and **GEDTA**), were synthesized in several steps from the parent CDs, *via* primary side per[bis(carboxymethy)amino] substitution. Complexation of EDTA-CDs with lanthanide ions was a very efficient process, as shown by spectroscopic methods. **AEDTA** and **BEDTA** form coordination complexes with up to three lanthanide ions, whereas four ions coordinate with the larger **GEDTA**. The average value of inner-sphere water molecules is 1.5 for **BEDTA** complexes. The molecular relaxivity values of the Gd(III) complexes with the EDTA-CDs at 12 MHz were substantially higher than those of

small commercial contrast agents, and even higher at 100 MHz, due to the beneficial effect of the CDs on the rotational correlation time and to the presence of several paramagnetic centers per macrocyclic ligand. In blood plasma, the complexes still gave very high relaxivity values, especially those of **GEDTA**. The Gd(III) complexes were found to be non-toxic in cell toxicity tests. Semiemprirical PM3 quantum-mechanical calculations, aided by benchmark DFT calculations for model complexes, were carried out for the first time to the best of our knowledge, on molecular systems of this size containing lanthanide atoms. The results indicate that the CDs are flexible enough to allow efficient coordination of 3 and up to 4 lanthanide ions, located in the periphery of the primary side away from the CD cavity, together with approximately two exchangeable water molecules. The theoretical findings are in good agreement with the experimental results and they can justify the inability of the macrocycles to exhibit supramolecular sensitization of the lanthanide luminescence by appropriate guests.

The above outlined properties of the EDTA-type CDs along with their aqueous solubility and low toxicity suggest that the corresponding Gd(III) complexes can be used as contrast agents in NMR imaging applications. Additional anticipated properties include minimal potential interaction with anionic proteins and membranes *in vivo* and a long circulation time before excretion. There is great current interest in the development of MRI contrast agents,[47] not only regarding Gd(III) with its fast exchanging coordinated water molecules, but also regarding Eu(III) coordinated with slow exchanging water molecules, utilized in paramagnetic chemical exchange saturation transfer (PARACEST) sequences, metabolic MRI agents, targeted agents *etc*. Thus, development of new and versatile molecular imaging probes with possibility to use them in combined imaging modalities can be of great experimental and clinical importance.

Experimental

Water was purified by the Purelab Plus system (USF ELGA). Dimethylformamide (DMF) was distilled over molecular sieves. Derivatives **1**,[23] **2**,[23] **3(a,b)**[25] and **4(a,b)**[25] were prepared according to the published methods. The pH was measured using a MP200 Mettler Toledo pH meter. High resolution one- and two-dimensional NMR spectra were acquired at 500 MHz on a Bruker AVANCE spectrometer using standard pulse sequences. ^{1}H NMR spectra recorded in D_2O were referenced to HOD at δ 4.79. Spectrofluorimetric titrations were carried out by adding the ligands (10 µl of 15 mM solution) to a solution (1 mM) of $EuCl_3$ or $TbCl_3$ in HEPES buffered H_2O (pH 7.0). The additions stopped upon formation of a precipitate. During the titration the maximum intensity of the emissions [Eu(III), 617 nm; Tb(III), 545 nm] was monitored, the excitation wavelengths being 394 nm for Eu(III) and 368 nm for Tb(III). Corrections for dilutions were appropriately applied. UV-Visible absorption spectra were recorded using a Perkin-Elmer Lambda-16 spectrometer using quartz cuvettes of 1 cm path length. Luminescence spectra and lifetimes were measured using a Perkin-Elmer LS 50B instrument using a 1 × 1 cm quartz cell. The excitation and emission band passes were 10 nm. IR spectra (KBr) were recorded on Perkin-Elmer System 2000 FT-IR instrument, run with FT-IR Spectrum v2.00 software.

ESI-TOF MS experiments were performed on a time-of-flight mass analyzer equipped with electrospray interface (Bruker, MicrOTOF) operating in positive mode in the mass range from 300 to 2000 *m/z* (Spray voltage -4.0kV; N_2 nebuliser gas: 0.4 bar; N_2 dry gas: 4 L min^{-1}; dry gas temperature: 200 °C). Sample solution was introduced in the ionization source at 4 µL min^{-1} by a syringe pump. MALDI-TOF spectra were recorded on an Autoflex Bruker mass spectrometer, operating in linear or reflectron mode and in positive and/or negative polarity, according to the analyte structure. Matrix solution was prepared using 2,5-dihydroxybenzoic acid (DHB) at a concentration of 10 mg ml^{-1} in 80% ethanol. The Matrix-Analyte mixture prepared at the ratio 5 : 1 (v/v) was spotted (1 µL) on the MALDI target and left to air-dry at room temperature.

Hexakis[6-bis(*t*-butyloxycarbonylmethyl)amino-6-deoxy-2,3-di-*O*-methyl]-αCD (5a)

t-Butyl bromoacetate (0.245µL, 1.63 mmol) was added to a solution of hexakis(6-amino-6-deoxy-2,3-di-*O*-methyl)-αCD (**4a**, 0.077 g, 0.067 mmol) in acetonitrile (5 mL), together with potassium carbonate (0.225 g, 1.63 mmol) and potassium iodide (catalytic amount). The mixture was stirred under a N_2 atmosphere for 4 days at reflux (bath temperature 82 °C). After removal of the solvent, the residue was taken up into a mixture of dichloromethane and water. The organic layer was separated and the aqueous solution extracted with dichloromethane (2 × 10 mL). The organic extracts were combined, dried over magnesium sulfate and the solvent removed under reduced pressure to give a pale yellow solid. Purification was achieved by column chromatography on silica gel (gradient elution from CH_2Cl_2 to 10% MeOH–CH_2Cl_2, R_f 0.5 in 5% MeOH–CH_2Cl_2), to give compound **5a**; yield 0.16 g (56%). δ_H (500 MHz; $CDCl_3$; 298 K) 5.0 (s, 6H, H1), 4.15 (t, J = 8.9 Hz, 6H, H4), 3.56 (s, 18H, CH_3), 3.49 (brs, 6H, H3), 3.41 (s, 18H, CH_3), 3.35-3.32 (m, 24H, H7, H8), 3.20-3.05 (m, 12H, H2, H6). δ_C (125 MHz; $CDCl_3$) 171.3 (C=O), 99.7 (C1), 82.3 (C2), 81.8 (C3), 81.15 (C4), 80.3 (*C*-*t*Bu), 73.7 (C5), 61.8 (CH_3), 58.4 (CH_3), 57.5 (C7, C8), 40.4 (C6), 28.4 (*tBu*). MS (MALDI-TOF) *m/z* 2526.4 ([M+Na]$^+$, calcd: 2526.41). (Found: C, 55.9; H, 8.3; N 3.5. $C_{120}H_{210}O_{48}N_6 \cdot 3H_2O$ requires C, 56.3; H, 8.5; N, 3.3%).

Hexakis[6-bis(carboxymethyl)amino-6-deoxy-2,3-di-*O*-methyl]-αCD (AEDTA)

Trifluoroacetic acid (100 equiv.) was added to a solution of the *t*-butyl ester **5a** (50 mg, 0.02 mmol) in chloroform (5 ml) under N_2 atmosphere. After stirring at rt for 3 days, the solvent was removed under reduced pressure and the solid was washed with more dichloromethane (3 × 5 mL). Water was then added and the pH was raised to 7.0 using aqueous NaOH (1 M). The solution was placed directly into a dialysis membrane and dialyzed for 3 days (the external water was renewed every 24 h). The solvent was removed under reduced pressure to give a brownish residue. Yield 30 mg (71%). δ_H (500 MHz; D_2O; 298 K) 5.38 (s, 6H, H1), 4.41 (brs, 6H, H5), 3.97-3.93 (m, 30H, H3, H7, H8), 3.85-3.75 (m, 6H, H6), 3.60 (s, 18H, CH_3), 3.55 (s, 18H, CH_3), 3.31-3.47 (m, 18H, H2, H4, H6′). δ_C (125 MHz; D_2O) 170.5 (C=O), 97.3 (C1), 79.3 (C2, C3), 78.8 (C4), 68.4 (C5), 59.6 (CH_3), 59.2 (CH_3), 58.1 (C7, C8), 56.2 (C6). MS (MALDI-TOF) *m/z* 1853.7 ([M+Na]$^+$,

calcd for $C_{72}H_{114}N_6O_{48}Na$: 1853.66). (Found: C, 38.3; H, 5.6; N, 3.7. $C_{72}H_{102}N_6O_{48}Na_{12}8H_2O$ requires C, 38.6; H, 5.3; N, 3.75%).

Heptakis[6-bis(*t*-butyloxycarbonylmethyl)amino-6-deoxy-2,3-di-*O*-methyl]-βCD (5b)

t-Butyl bromoacetate (0.842 g, 4.2 mmol) was added to a solution of heptakis(6-amino-6-deoxy-2,3-di-*O*-methyl)-βCD (**4b**, 0.2 g, 0.15 mmol) in acetonitrile, together with potassium carbonate (0.58 g, 4.2 mmol) and potassium iodide (0.1 g, 0.6 mmol). The solution was stirred under a N_2 atmosphere for 5 days at reflux. After removal of the solvent, the residue was taken up into a mixture of dichloromethane (100 mL) and aqueous NaOH solution (1M, 100 mL); the organic layer was separated and the aqueous solution extracted with dichloromethane (3 × 100 mL). The extracts were combined, dried over sodium carbonate and the solvent removed under reduced pressure to give a pale yellow solid. Purification was achieved by column chromatography on silica gel (gradient elution from CH_2Cl_2 to 10% MeOH–CH_2Cl_2, R_f 0.5 in 5% MeOH–CH_2Cl_2), to give compound **5b**; yield 0.32 g (73%). δ_H (500 MHz; $CDCl_3$; 298 K) 5.16 (s, 7H, H1), 3.98-3.95 (t, J = 9.35 Hz, 7H, H4), 3.69-3.67 (m, 7H, H5), 3.55 (s, 21H, CH_3), 3.52-3.50 (m, 7H, H3), 3.45 (s, 21H, CH_3), 3.38-3.34 (m, 28H, H7, H8), 3.20-3.02 (m, 14H, H2, H6). δ_C (125 MHz; $CDCl_3$; 298 K) 171.08 (C=O), 98.9 (C1), 82.1 (C2), 81.6 (C3), 81.0 (C4), 80.3 (*C*-*t*Bu), 73.2 (C5), 61.4 (CH_3), 58.3 (CH_3), 57.9 (C7, C8), 55.1 (C6), 28.4 (*tBu*). MS (ESI-TOF) m/z 1484.3 ($[M+2Na]^{2+}$, calcd: 1484.1). (Found: C, 57.8; H, 8.5; N, 3.05. $C_{140}H_{245}O_{56}N_7$ requires C, 57.5; H, 8.45; N, 3.4%).

Heptakis[6-bis(carboxymethyl)amino-6-deoxy-2,3-di-*O*-methyl]-βCD (BEDTA)

Trifluoroacetic acid (100 equiv.) was added to a solution of the *t*-butyl ester **5b** (200 mg, 0.068 mmol) in chloroform (5 ml) under a N_2 atmosphere. After stirring at 25 °C for 2 days, the solvent was removed under reduced pressure and the solid was washed with more dichloromethane (3 × 5 mL), the solvent being removed each time. Water was then added and the pH was raised to 7.0 using aqueous NaOH (1 M). The solution was placed directly into a dialysis membrane and dialyzed for 3 days (the external water was renewed every 12 h). The solvent was removed under reduced pressure to give a white solid. Yield 96 mg (66%). δ_H (500 MHz; D_2O) 5.39 (s, 7H, H1), 4.36 (brs, 7H, H5), 3.95 (brs, 35H, H3, H7, H8), 3.91-3.84 (m, 7H, H6), 3.60-3.55 (m, 63H, CH_3×2, H2, H4, H6′). δ_C (125 MHz; D_2O) 170.5 (C=O), 97.02 (C1), 79.5 (C2, C3, C4), 68.1 (C5), 59.2 (CH_3), 58.8 (CH_3), 58.5 (C7, C8), 56.0 (C6). MS (MALDI-TOF) m/z 2158.8 ($[M+Na]^+$, calcd for $C_{84}H_{132}N_7O_{56}Na$: 2158.99. (Found: C, 40.4; H, 5.6; N, 3.8. $C_{84}H_{119}O_{56}Na_{14}N_7{\cdot}4H_2O$ requires C, 40.1; H, 5.1; N, 3.9%).

Octakis(6-azido-6-deoxy-2,3-di-*O*-methyl)-γCD (3c)[48]

Octakis(6-azido-6-deoxy)-γCD (**2**, 0.263 g, 0.175 mmol) in THF (6 mL) was added dropwise to a solution of NaH (0.170 g, 7.083 mmol) in THF at 0 °C. The mixture was left stirring for 30 min and then MeI (3 mL) was added. The reaction mixture was left at 0 °C for 1 h and at rt for 16 h always protected from light. The remaining NaH was neutralized with MeOH (6 mL). After removal of the solvent, the residue was taken up into a mixture of dichloromethane (20 mL) and brine (20 mL); the organic layer was separated and the aqueous solution extracted with dichloromethane (3 × 20 mL). The extracts were combined, dried over $MgSO_4$ and the solvent removed under reduced pressure to give a pale yellow solid; yield 0.267 g (88%). δ_H (500 MHz; $CDCl_3$; 298 K) 5.22 (brs, 8H, H1), 3.74 (brs, 8H, H5), 3.65 (m, 8H, H6), 3.57 (s, 24H, CH_3), 3.50 (m, 24H, H6′, H3, H4), 3.47 (s, 24H, CH_3), 3.14 (m, 8H, H2). δ_C (125 MHz; $CDCl_3$; 298 K) 98.02 (C1), 81.7 (C4, C3), 79.4 (C2), 70.9 (C5), 61.28 (CH_3), 59.06 (CH_3), 51.63 (C6-N_3).

Octakis(6-amino-6-deoxy-2,3-di-*O*-methyl)-γCD (4c)[49]

PPh_3 (0.742 g, 2.829 mmol) was added to a solution of octakis(6-azido-6-deoxy-2,3-di-*O*-methyl)-γCD (**3c**, 0.267 g, 0.155 mmol) in DMF (6 mL). The mixture was left for 1.5 h and then aqueous NH_3 30% was added drop wise. The reaction mixture was left at rt for 16 h. After removal of the solvent, pH was adjusted to 4 with 0.5 M HCl and the residue was taken up into a mixture of dichloromethane (20 mL) and H_2O (20 mL); the organic layer was separated and the aqueous solution was evaporated under reduced pressure to give a pale yellow solid; yield 0.234 g (100%). δ_H (500 MHz; D_2O; 298 K) 5.42 (brs, 8H, H1), 4.11 (brs, 8H, H5), 3.74 (m, 24H, H2, H3, H4), 3.49 (s, 24H, CH_3), 3.48 (s, 24H, CH_3), 3.38 (m, 8H, H6), 3.24 (ap.t, 8H, H6′). δ_C (125 MHz; D_2O; 298 K) 96.5 (C1), 79.5 (C4, C3, C2), 67.3 (C5), 59.13 (CH_3×2), 40.7 (C6-NH_2).

Octakis[6-bis(*t*-butyloxycarbonylmethyl)amino-6-deoxy-2,3-di-*O*-methyl]-γCD (5c)

t-Butyl bromoacetate (1.237 g, 6.34 mmol) was added to a solution of octakis(6-amino-6-deoxy-2,3-di-*O*-methyl)-γCD (**4c**, 0.3 g, 0.198 mmol) in acetonitrile, together with potassium carbonate (0.876 g, 6.34 mmol) and potassium iodide (catalytic). The solution was stirred under nitrogen for 4 days at reflux (bath temperature 82 °C). After removal of the solvent, the residue was taken up into a mixture of dichloromethane (50 mL) and H_2O (50 mL); the organic layer was separated and the aqueous solution extracted with dichloromethane (3 × 50 mL). The extracts were combined, dried over $MgSO_4$ and the solvent removed under reduced pressure to give a pale yellow solid. Purification was achieved by column chromatography on silica gel (gradient elution from CH_2Cl_2 to 5% MeOH–CH_2Cl_2, R_f 0.2 in 5% MeOH–CH_2Cl_2), to give compound **5c**; yield 0.299 g (45%). δ_H (500 MHz; $CDCl_3$; 298 K) 5.38 (s, 8H, H1), 4.05 (m, 8H, H4), 3.70 (m, 8H, H5), 3.62 (s, 24H, CH_3), 3.53 (m, 16H, H7), 3.52 (s, 24H, CH_3), 3.49 (m, 24H, H3, H8), 3.28 (brs, 8H, H6), 3.12 (m, 28H, 8H, H2), 3.11 (m, 8H8, H6′). δ_C (125 MHz; $CDCl_3$) 170.8 (C=O), 98.39 (C1), 82.0 (C2, C3), 80.46 (*C*-*t*Bu), 79.9 (C4), 72.9 (C5), 61.59 (CH_3), 58.7 (CH_3), 57.45 (C7, C8), 55.18 (C6), 28.39 (*tBu*). MS (MALDI-TOF) m/z 3361.9 ($[M+H^+Na]^+$, 100%), calcd for $C_{160}H_{280}N_8O_{64}NaH$: 3361.89. (Found: C, 56.4; H, 8.5; N, 3.3. $C_{160}H_{280}N_8O_{64}{\cdot}3H_2O$ requires C, 56.6; H, 8.5; N, 3.3%).

Octakis[6-bis(carboxymethyl)amino-6-deoxy-2,3-di-*O*-methyl]-γCD (GEDTA)

Trifluoroacetic acid (114 equiv,.0968 g, 8.498 mmol) was added to a solution of the *t*-butylester **5c** (249 mg, 0.0745 mmol) in chloroform (7 ml) under Ar atmosphere. After stirring at rt for

2 days, the solvent was removed under reduced pressure and the solid was washed with more dichloromethane (3 × 5 mL), the solvent being removed each time. Water was then added and the pH was raised to 7.0 using aqueous NaOH (1 M). The solution was placed directly into a dialysis membrane and dialyzed for 4 days (the external water renewed every 12 h). The solvent was removed under reduced pressure to give a white solid. Yield 91 mg (50%). δ_H (500 MHz; D_2O; 298 K) 5.40 (s, 8H, H1), 4.27 (brs, 8H, H5), 3.88 (m, 32H, H7, H8), 3.85 (m, 8H, H3), 3.73 (m, 8H, H6′), 3.62 (s, 24H, CH_3), 3.57 (s, 24H, CH_3), 3.51 (m, 16H, H2, H4), 3.49 (m, 8H, H6). δ_C (125 MHz; D_2O) 170.4 (C=O), 96.83 (C1), 79.7 (C4), 79.67 (C3), 79.6 (C2), 67.8 (C5), 59.53(CH_3), 59 (CH_3, C7, C8), 55.96 (C6). MS (MALDI-TOF) *m/z* 2463.8 ([M+Na]$^+$, 100%), 2486.8 ([M+2Na]$^+$, 66%), calcd for $C_{96}H_{152}N_8O_{64}$ Na: 2463.89. (Found: C, 40.5; H, 5.8; N, 3.8. $C_{96}H_{138}N_8O_{64}Na_{14}.6H_2O$ requires C, 40.3; H 5.3; N, 3.9%).

Formation of complexes

The lanthanide(III) complexes were obtained from the respective ligands by addition of the appropriate equivalent amount of $EuCl_3$ or $TbCl_3$ or $Gd(NO_3)_3$ in water followed by 1 h stirring under a N_2 atmosphere.

Relaxivity measurements

The T_1 relaxation times for Gd(III) chelates with **AEDTA**, **BEDTA** and **GEDTA** at five concentrations (from 6 mM to 30 mM in Gd(III) ions or 3 mM to 15 mM in Gd(III) ions for AEDTA) in 200 μL aqueous solutions were determined on a BRUKER MSL-100 spectrometer at 0.29 T (12 MHz) and 2.35 T (100 MHz) using a portable Halbach magnet and a superconducting magnet, respectively. All measurements were performed at 25 °C. T_1 was measured using the standard inversion recovery technique,[50] with repetition time $T_R = 10\,000$ ms, for inversion times τ between 0.5 and 10 000 ms. The recovery data were fitted to a three parameter exponential recovery function using a non-linear least-squares fitting routine. The relaxivities, r_1, in mM^{-1} s^{-1} were evaluated from the slopes of $1/T_1$ *versus* molar concentration of solutions using a least-squares fitting routine. The blood plasma was obtained after removal of the blood cells by centrifuge of blood drawn from a healthy individual. The complexes were prepared as in the case of water and T_1 measurements at the different Gd(III) concentrations were carried out within 2.5 h as above.

Cells and culture conditions

The human skin fibroblast strain AG01523c was obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). Cells were routinely cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with antibiotics and 10% Fetal Bovine Serum (FBS) in an environment of 5% CO_2, 85% humidity and 37 °C, and they were subcultured using trypsin–citrate (0.25–0.3%, respectively) solution at a 1 : 2 split ratio. Cells were tested and found to be mycoplasma-free. All cell culture media were from Gibco-BRL (Paisley, UK).

MTT assay. Cytotoxicity was evaluated by a modification of the MTT assay.[41] Cells were plated in 96-well flat-bottomed microplates at a density of approximately 7000 cells per well. 18 h after the plating, the culture medium was replaced with serial dilutions of the suspensions under study in fresh medium. After a 72 h incubation, the cells were washed with phosphate buffered saline (PBS) to remove any residual traces of the suspension and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) dissolved at a final concentration of 1 mg mL^{-1} in serum-free, phenol-red-free DMEM (Biochrom KG) was added for a further 4 h incubation. Then, the MTT-formazan was solubilized in isopropanol and the optical density was measured at a wavelength of 550 nm and a reference wavelength of 690 nm.

Computational procedure

All theoretical calculations for substituted cyclodextrins and their metal complexes were performed by the MOPAC 2009 program.[51] The PM3 hamiltonian[52] was used for all elements with the exception of the tripositive lanthanide cations La^{+3}, Eu^{+3}, Gd^{+3}, Tb^{+3} and Lu^{+3}, which were treated as sparkles using AM1 parameters.[53,54] Geometry optimization was accomplished by the Eigenvector Following approach (EF) using optimization convergence criteria corresponding to a maximum gradient norm of 0.05 kcal mol^{-1} $Å^{-1}$. The DFT calculations were performed using the Gaussian 98 suite of programs.[55] The B3P86 functional[56,57] was employed in conjunction with the 3-21G* basis set [58,59] for the H, C, N, O and S atoms, and has been shown to yield reliable geometric parameters for organic molecules,[60] whereas a quasi-relativistic pseudopotential was employed for lanthanide atoms which incorporates their 4f electrons into core-functions,[61] leaving a valence space of 11 electrons for the neutral atoms.

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