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Macrocyclic trinuclear gadolinium(III) complexes: the influence of the linker flexibility on the relaxometric properties

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Multimeric systems assembled by linking Gd^{III} complexes to a central scaffold can be Magnetic Resonance Imaging (MRI) contrast agents of improved efficiency at high magnetic fields. Two novel mononuclear GdDO3A-derivatives (DO3A = 1,4,7,10-tetraazacyclododecane-*N*,*N'*,*N"*-triacetic acid) featuring a flexible (hexanoic acid, Gd**L1**) or rigid (methyl benzoic acid, Gd**L2**) pendant arm were synthesized and conjugated to a central 1,4,7-triazacyclononane unit through amide coupling to form two novel trinuclear systems. A variable temperature and frequency ^{1}H and ^{17}O NMR relaxometric study on the mononuclear complexes indicated that Gd**L2** exhibits two water molecules of in the inner coordination sphere, as for the parent $[Gd(DO3A)(H_2O)_2]$ complex (q = 2). Instead, the flexibility of the alkyl chain of Gd**L1** allows its folding and coordination to the Gd^{3+} ion with displacement of one water molecule (q = 1) around neutral pH. In Gd_3**L4**, the linker rigidity results in a compact and rather rigid trinuclear system from the point of view of rotational dynamics, with each Gd^{III} center having q = 2. This favors a considerable degree of coupling between local and overall tumbling motions and hence high relaxivity values (r_1 = 13.8 mM^{-1} s^{-1}; 60 MHz and 298 K). On the other hand, the flexibility of the alkyl chain significantly affects the properties of Gd_3**L3**. The relaxation data suggest the easy folding of one of the chelates with coordination of Gd by an amide group of the central macrocyclic unit (q = 1). The other two complexes (q = 2) exhibit a high degree of rotational mobility around the linker that results in a significant limit on the relaxivity (r_1 = 9.8 mM^{-1} s^{-1}; 60 MHz and 298 K).

Introduction

Complexes of paramagnetic metal ions, especially Gd(III) and more recently Mn(II), are used daily as contrast agents (CAs) in a large proportion of clinical and pre-clinical studies with magnetic resonance imaging (MRI).[1] Despite the widespread use, their performance (relaxivity, r_1) is far from optimal, leaving a wide margin for further development and improvement. Two are the fundamental approaches to obtain high performance MRI probes: i) optimization of the relaxation properties of the individual metal complexes; ii) combining a number of paramagnetic units together in multimeric or nanosized systems.[2] The successful pursuit of these two approaches is one of the ultimate goals in MRI contrast agent development for Molecular Imaging applications.[3]

In recent years, two key parameters have been more thoroughly studied because they most significantly affect the efficacy of the MRI probes. One is the rotational correlation time, τ_R, which characterizes the tumbling motion of the complex and is correlated to its molecular size and stereochemical rigidity. The other is the water exchange between the paramagnetic metal site and bulk water, k_{ex} = $1/\tau_M$ (τ_M = mean residence lifetime). The water-exchange rates measured for Ln^{III} complexes cover a remarkable wide range of values over more than six orders of magnitude, roughly from 10^9 to 10^3 s^{-1}. Optimal τ_M values are rather short for T_1-shortening agents, approximately in the range of 1 to 100 ns for rapidly tumbling Gd^{III} or Mn^{II} complexes. The Solomon-Bloembergen-Morgan (SBM) model of paramagnetic relaxation[4] shows that high relaxivities can be obtained by increasing τ_R, but only in the case of low and intermediate magnetic fields (< 2 T). At high (about 3T) or ultra-high (> 3T) magnetic fields, there is an optimal value of τ_R that depends on the applied magnetic field strength.[5] Furthermore, relaxivity can be further enhanced by increasing the number of coordinated (inner sphere) water molecules (q).

Because of technological developments, higher field magnets are increasingly utilized in clinical or pre-clinical studies (3-7 T for clinical applications and up to 17.6 T for animal studies). High fields MRI imaging has several advantages, as it amplifies the sensitivity and increases the spatial/temporal resolution. These technological advances are accompanied by an intense research to optimize the properties of MRI probes for use at high frequencies.[1,5]

Optimal high field CA candidates are paramagnetic systems with intermediate molecular weight (in the range ca. 1-4 kDa), corresponding to τ_R values between 0.3-1 ns.[5] One possible strategy to meet these conditions consists in combining a suitable number of Gd complexes to a central scaffold. The

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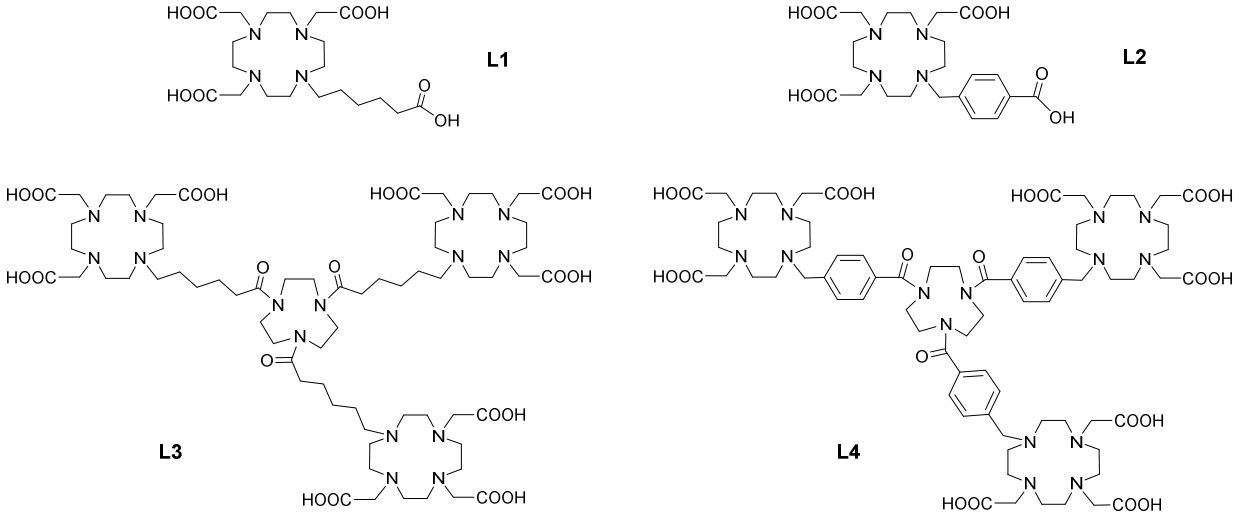
design of the metallostar complex represents one of the pioneering work along this direction. It consists of a supramolecular structure based on the self-assembly of dimeric



Scheme 1. Chemical structures of ligands L1-L4.

bishydrated $(GdDTTA)_2$-bipyridine (DTTA = diethylenetriamine-*N*,*N*,*N*",*N*"-tetraacetate) complexes around a Fe(II) complex core.[6] Metallostar has a remarkable relaxivity at high proton Larmor frequencies as a result of the presence of six q = 2 Gd-chelates, an intermediate molecular weight and high motional rigidity. Encouraging results were obtained in the case of trinuclear complexes in which a central benzene unit was bound with three methylene DTTA moieties[7] or three DO3A complexes.[8] Also GdAAZTA (AAZTA = 6-amino-6-methylperhydro-1,4-diazepine tetraacetate) has been attached to a small dendritic core to obtain tetranuclear or octanuclear systems with high relaxivity at high magnetic fields.[9,10] Moreover, it is worthwhile to highlight that, for a safe use as CAs, paramagnetic complexes need to be characterized by high thermodynamic stability and kinetic inertness under physiological conditions, hence macrocyclic or mesocyclic complexes should be preferred.

In this work we have linked bishydrated macrocyclic GdDO3A units to a macrocyclic scaffold based on the 1,4,7-triazacyclononane core. Two different spacers were used, one consisting of a flexible alkyl chain based on hexanoic acid and another shorter and compact, and hence less flexible, based on 4-methyl benzoic acid (Scheme 1). The core unit features three secondary amine groups that enables the development of trinuclear complexes, while the two spacers provide the complexes with different properties in terms of rotational dynamics. Thus, we report herein the synthesis of two novel ligands and their mononuclear and trinuclear Gd(III) complexes. A complete ^{1}H and ^{17}O NMR relaxometric study on the paramagnetic systems has allowed the evaluation of the relaxation parameters and has provided useful information on their dependence on molecular geometry and structure. Important insights have been obtained on the influence of the nature of the linker on the proton longitudinal relaxation properties of the trinuclear complexes at the frequencies relevant to MRI applications.

Results and discussion

Synthesis

The synthesis of the monomeric DO3A-based ligands **L1** and **L2** was accomplished in three steps by modification of published literature procedures.[11,12] In detail, the syntheses started by alkylation of DO3A tris-*t*Bu ester with the corresponding methyl alkanoate (methyl 6-bromohexanoate or methyl p-bromomethylbenzoate) followed by methyl ester hydrolysis and *t*Bu ester deprotection. For the synthesis of the trimers, the tri-amine 1,4,7-triazacyclododecane (TACN) was used as central scaffold to attach three monomeric units. TACN is seldom used as scaffold for the synthesis of multimeric ligands, for example to link three targeting vectors[13] or to construct a multifunctional system by exploiting the central coordinating unit like in TRAP systems (TRAP = triazacyclononane-phosphinic acid).[14] Our trimers were synthesised by amide coupling of the pendant carboxylic acid of the monomeric ligands with the macrocyclic tri-amine. The lower coordinating ability of the amides should prevent the tris-amide macrocyclic scaffold to interfere with the Gd^{III} coordination. The final ligands were obtained by *t*Bu ester deprotection with a mixture of trifluoroacetic acid in dichloromethane. All of the compounds were characterized by

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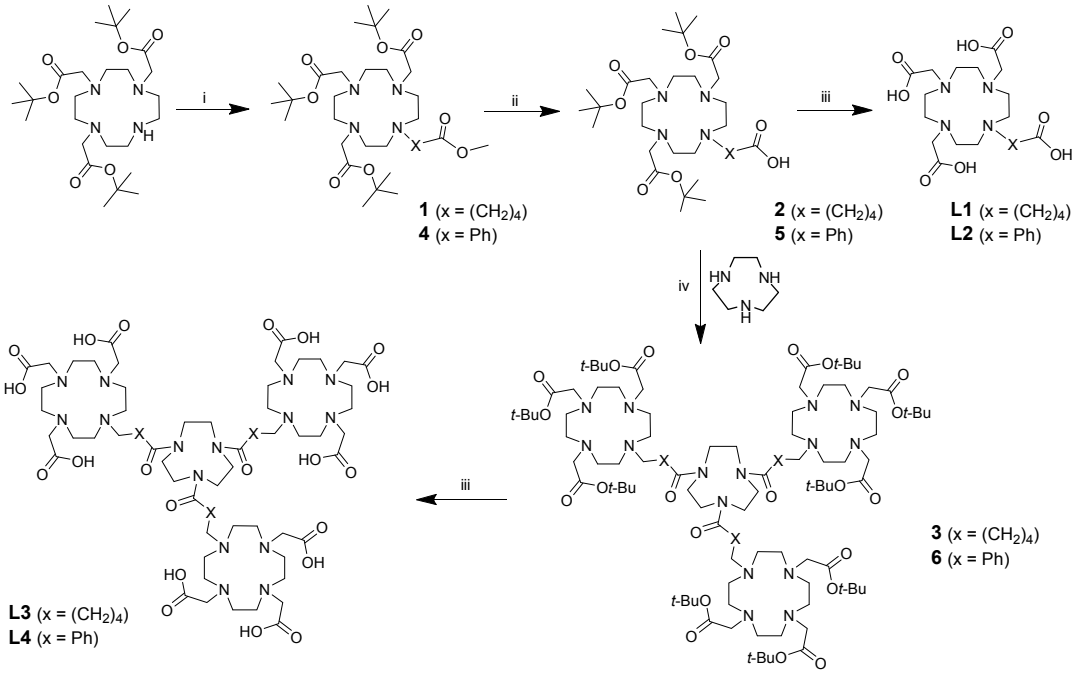

Scheme 2. Synthesis scheme for ligands L1-L4: i) alkylbromide (methyl 6-bromohexanoate or methyl p-bromomethylbenzoate), CH_3CN, K_2CO_3, KI; ii) LiOH, MeOH, H_2O; iii) TFA/CH_2Cl_2 1:1; iv) HATU, DMF, NEt_3.

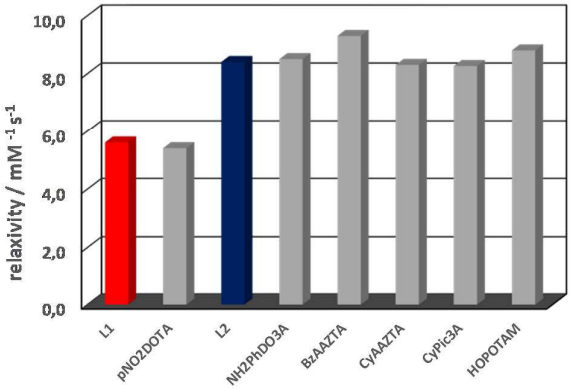

Figure 1. r_1 values of Gd**L1** and Gd**L2** (20 MHz, 298 K) compared to those of other q = 1 or q = 2 Gd^{III} complexes of similar molecular size and measured under identical experimental conditions.[15-20]

ESI mass spectrometry and ^1H and ^{13}C NMR spectroscopy. The Gd^{III} complexes were prepared at room temperature by adding $Gd(NO_3)_3$ to a solution of **L1**-**L4** while maintaining the pH at 6.5 with diluted NaOH. The excess of free metal ions in the solution was precipitated by the addition of NaOH up to pH 9 and a successive centrifugation, filtration and lyophilisation yielded white solids corresponding to the Gd-complexes.

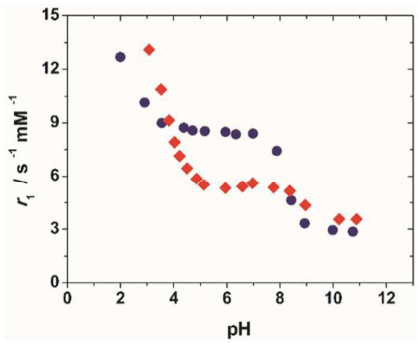

Figure 2. r_1 values of Gd**L1** (red diamonds) and Gd**L2** (blue circles) as a function of pH (20 MHz, 298 K).

Relaxometric characterization

A) GdL1 and GdL2. The relaxivity values of Gd**L1** and Gd**L2** were measured to be 5.6 and 8.4 mM^{-1} s^{-1} at 20 MHz, respectively (298 K, pH 7). A 50% difference in r_1 values suggests that the two complexes are characterized by a different hydration state. In fact, while the relaxivity of Gd**L1** is quite typical of the low-molecular weight q = 1 Gd^{III} complexes, that of Gd**L2** is consistent with the presence of two water

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molecules coordinated to the paramagnetic metal ion (q = 2) in agreement

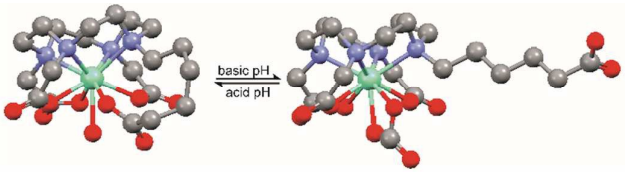

Figure 3. Schematic representation of the pH dependent hydration equilibrium of Gd**L1**. Left: acid pH and q = 1; right: CO_3^{2-} coordination and q = 0.

with the corresponding values measured under identical experimental conditions for other bishydrated Gd^{III} complexes of similar molecular size (Figure 1).[15-20] A further support for the different values of the hydration number q of the two complexes derives from the study of the pH dependence of r_1. Typically, bishydrated complexes such as GdDO3A and derivatives exhibit a nearly constant value of r_1 in the range of pH ca. 4 - 8.5. At lower pH values, relaxivity increases following the progressive dissociation of the complex, while at more basic values a marked decrease of r_1 is observed due to the water displacement and formation of ternary complexes (q = 0) with carbonate anions present in the aerated solution.[21] We have observed a similar behaviour for Gd**L2**, for which r_1 assumes a constant value of 8.4 mM^{-1} s^{-1} between pH 4 and 8, followed by a marked decrease up to 2.9 mM^{-1} s^{-1} at pH = 10.5 (Figure 2). Gd**L1** also shows similar behaviour in the basic region, with a decrease of r_1 from 5.6 to ca. 3.5 mM^{-1} s^{-1} increasing the pH from 7 to 10. Therefore, for both complexes the value of relaxivity at basic pH indicates the presence of a species (largely prevalent) with q = 0. While the formation of ternary complexes with coordinating anions corresponding to Δq = 2 is quite common, the change of the hydration number from 1 to 0 is definitely less frequent.[21] The most plausible hypothesis that might accounts
for this behaviour is the involvement of the carboxylic group of the hexanoic chain in the coordination of the metal ion. This implies the formation of a monohydrated species around neutral pH. We propose that, due to the formation of an unstable nine-membered ring, the coordinated hexanoic moiety can be easily displaced by the carbonate anion leading to the formation of a ternary (q = 0) complex (Figure 3).
Noteworthy, the intramolecular coordination of the hexanoic pendant arm in Gd**L1** is in contrast to the recently reported formation of a GdDO3A-arylsulfonamide dimeric structure through intermolecular coordination of a peripheral carboxylate group. In that case, however, the pendant arm always involved the presence of a rigid phenyl ring that for steric reasons prevents the intramolecular coordination.[22]
The variation of r_1 as a function of the applied magnetic field strength, the so-called Nuclear Magnetic Resonance Dispersion profile (^{1}H NMRD), was measured at 298 K in the proton Larmor frequency range 0.01–70 MHz, corresponding to magnetic field strengths varying between 2.34×10^{-4} and 1.64 T (Figure 4). The NMRD curves of Gd**L1** and Gd**L2** are characterised by identical shape and different amplitude, which confirm that the different hydration states of the two complexes are largely responsible for their different relaxing efficiencies. In the low field region (0.01-2 MHz) the profiles exhibit a constant value of r_1, followed by a single dispersion centred about 6-8 MHz, quite typical of rapidly tumbling complexes, and then by another plateau with lower relaxivity in the high-frequency region (> 30 MHz). The ratio between the r_1 values at 0.01 and 60 MHz for the two complexes varies only marginally to suggest similar values of the electronic relaxation ($T_{1,2e}$) and rotational correlation times (τ_R).

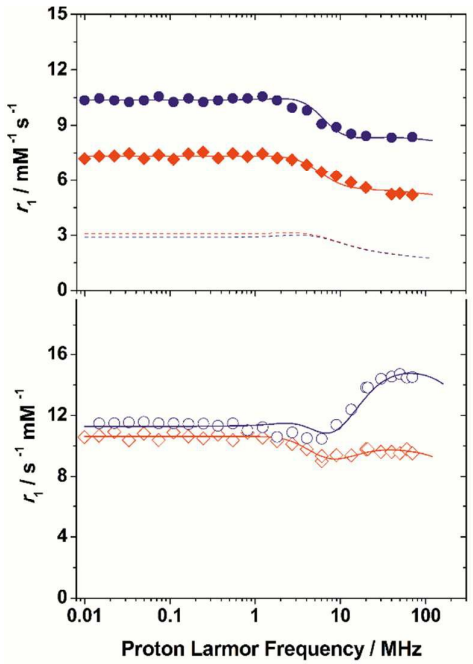

Figure 4. $1/T_1$ ^{1}H NMRD relaxation data measured at pH = 7.4 and 298 K. Top: Gd**L1** (red filled diamonds) and Gd**L2** (blue filled circles); bottom: Gd_3**L3** (red empty diamonds) and Gd_3**L4** (blue empty circles). The solid lines show the curves calculated using the best-fit parameters (see Table 1).

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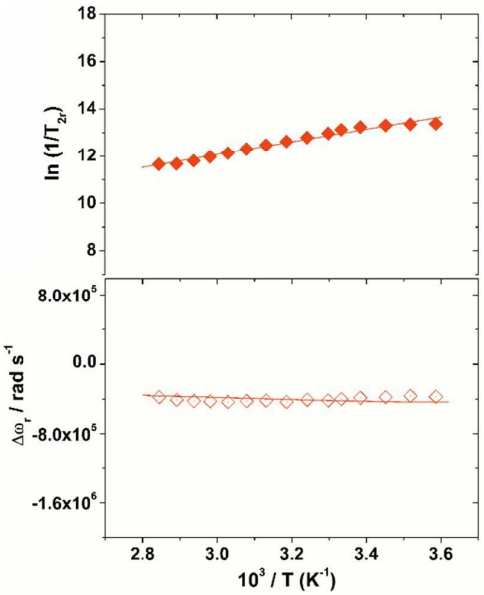

Figure 5. Temperature dependence of the reduced water ^{17}O NMR transverse relaxation rates (top) and chemical shifts (bottom) at 11.75 T and pH = 7 for an aqueous solution (18 mM) of Gd**L1**.

Table 1. Best-fit parameters obtained from the analysis of the $1/T_1$ ^{1}H NMRD profiles collected at 298 K and ^{17}O NMR data for Gd**L1**, Gd**L2**, Gd_3**L3** and Gd_3**L4** compared to other mononuclear or trinuclear Gd-complexes (Gd*p*NO_2DOTA[15], GdMBzDO3A[16], GdCyAAZTA[18] and Gd_3MesDO3A[8]).[a]

Parameter	Gd**L1**	Gd*p*NO_2DOTA	Gd**L2**	GdMBzDO3A	GdCyAAZTA	Gd_3**L3**	Gd_3**L4**	Gd_3MesDO3A
$^{20}r_1$ (mM^{-1} s^{-1})	5.6 ± 0.1	5.4	8.4 ± 0.1	9.5	8.3	9.8 ± 0.1	13.8 ± 0.2	13.7
q	1	1	2	2	2	1.67	2	2
τ_{RG} (ps)	112 ± 4	81	104 ± 2	81.1	97	370 ± 41	300 ± 26	201
τ_{RL} (ps)	-	-	-	-	-	90 ± 7	110 ± 9	-
S^2	-	-	-	-	-	0.12 ± 0.03	0.39 ± 0.04	-
k_{ex} (× 10^7 s^{-1})	12.5 ± 1.1	1.0	6.7 ± 0.6	1.76	0.91	12.5[b]	6.7[b]	3.2
Δ^2 (10^{19} s^{-2})	4.7 ± 0.2	2.7	5.4 ± 0.3	5.7	1.0	2.9 ± 0.1	4.8 ± 0.2	1.9
τ_V (ps)	27 ± 1	7.4	28 ± 2	20.3	59	36 ± 3	32 ± 2	10.9
$\Delta H^{\#}_M$ (kJ mol^{-1})	20.4 ± 1.1	-	25.5 ± 0.7	36.2	27.8	-	-	25.8
$A/\hbar$ (10^6 rad/s)	-3.2 ± 0.1	-	-3.2 ± 0.2	-	-	-	-	-

[a] The parameters fixed in the fitting procedure are: r_{GdO} = 2.5 Å, r_{GdH} = 3.1 Å , a_{GdH} = 4.0 Å , $^{298}D_{GdH}$ = 2.5×10^{-5} cm^2 s^{-1}, E_R = 18 kJ mol^{-1}, Ev = 1 *k*J.

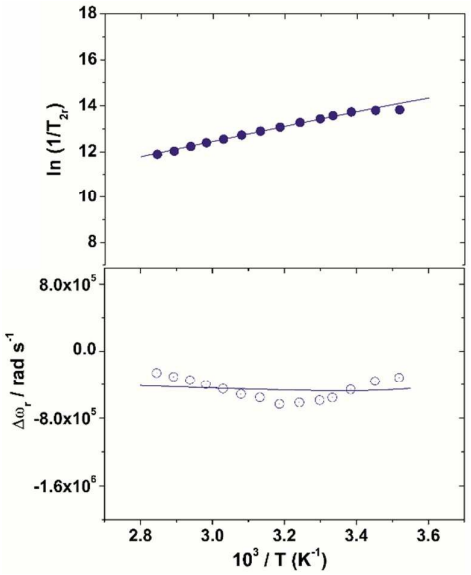

Figure 6. Temperature dependence of the reduced water ^{17}O NMR transverse relaxation rates (top) and chemical shifts (bottom) at 11.75 T and pH = 7.0 for an aqueous solution (7 mM) of Gd**L2**.

The temperature dependence of r_1 for both Gd**L1** and Gd**L2** shows an exponential increase with decreasing temperature over the range 5-60 °C, a behaviour typically observed in systems under the fast-exchange regime (Figure S1).[1] In this condition, r_1 is only limited by the rotational dynamics of the complexes and not by the rate of water(s) exchange, k_{ex}.

The measurement of the temperature dependence of the solvent ^{17}O NMR transverse relaxation rates, R_2, and shifts, $\Delta\omega$, represents the preferred procedure for obtaining information on the kinetics of water exchange because of its accuracy and reliability. The experiments were carried out on 18 and 7 mM solutions of Gd**L1** and Gd**L2**, respectively, at 11.75 T and neutral pH. The experimental data are reported as reduced transverse relaxation rates, R_{2r}, defined as $1/T_{2r} = R_{2r} = R_{2p}/p_M$, where p_M is the molar fraction of inner-sphere water molecules.[1a,6-8] The reduced transverse ^{17}O-relaxation rates and chemical shifts measured for Gd**L1** and Gd**L2** are reported in Figure 5 and 6, respectively. For both Gd^{III} complexes $1/T_{2r}$

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increases with decreasing temperature over the temperature range studied, indicating high rate of exchange for inner-sphere water molecule(s). The ^{17}O NMR data were analyzed in terms of the Swift-Connick theory[23] for ^{17}O relaxation, whereas the NMRD profiles were fitted according to the established theory of paramagnetic relaxation expressed in terms of the Solomon-Bloembergen-Morgan[4] and Freed's[24] equations for the *inner-* and *outer sphere* proton relaxation mechanisms, respectively. Given the large number of relaxation parameters, it is customary to fix some of them to known or typical values. The number q of coordinated water molecules was fixed to one for Gd**L1** and to two for Gd**L2**. The Gd-inner sphere water proton distance r and the distance of closest approach a of the *outer sphere* water molecules to Gd^{3+} were set to 3.1 and 4.0 Å, respectively, and for the relative diffusion coefficient D the standard value (at 298 K) of 2.24×10^{-5} cm^2 s^{-1} was used. In the fitting procedure, the following parameters were let to vary: the trace of the square of the transient zero-field splitting (ZFS) tensor Δ^2, the correlation time describing its modulation τ_V, the rotational correlation time τ_R, the mean residence lifetime of the inner sphere water molecule τ_M, its enthalpy of activation ΔH_M and the scalar Gd-$^{17}O_w$ coupling constant $A/\hbar$. The best-fit parameters are listed in Table 1 and compared with those of the parent GdDO3A and related mono- and bishydrated Gd complexes of similar size.

The rotational correlation time τ_R is sensibly longer (ca. 50%) than the value estimated for GdDO3A, reflecting the increase in the molecular mass due to the introduction of the pendant group. The k_{ex} value of 6.7×10^7 s^{-1} obtained for Gd**L2** (298 K) is one order of magnitude higher than that of GdDO3A, roughly twice as high the corresponding value of Gd(MBzDO3A)[11] and quite similar to that measured for GdHOPO-TAM.[20] It was previously shown that the rate of water exchange in neutral GdDO3A derivatives is modulated by the basicity of the macrocyclic nitrogen bearing the pendant group, with a faster water exchange associated with a greater basicity.[16] The present results confirm the previous hypothesis that the electronic properties of the substituent control the water exchange dynamics in this class of complexes. The rate of water exchange of Gd**L1**, $k_{ex} = 12.5\times10^7$ s^{-1}, is remarkably high for a neutral Gd chelate with q = 1 although not a unique example. For example, the k_{ex} value of the isomeric species of GdHPDO3A (q = 1) with a twisted square antiprimatic geometry is 11.2×10^7 s^{-1}.[25] In the case of Gd**L1**, the fast exchange of the coordinated water molecule can be tentatively associated with the high steric crowding of the bulky nine-membered ring, inducing a destabilization of the bound water molecule and thus a decrease in its mean residency lifetime, τ_M.

B) Gd_3L3 and Gd_3L4. The longitudinal proton relaxivity of the trinuclear complexes Gd_3**L3** and Gd_3**L4** are 9.8 and 13.8 mM^{-1} s^{-1}, respectively, at 20 MHz and 298 K. These values represent an increase of *ca.* 64 and 75% over the relaxivity of the corresponding mononuclear Gd**L1** and Gd**L2** complexes. The r_1 value for Gd_3**L4** is quite comparable with that of related bisaqua Gd-based trinuclear systems. For example, an almost identical relaxivity value (r_1 = 13.7 mM^{-1} s^{-1}, at 20 MHz and 298 K) was reported for the closely related neutral trinuclear [Mes{Gd(DO3A)$(H_2O)_2$}$_3$], featuring three DO3A units linked to the methyl positions of a central mesitylene.[8] Instead, a higher value (r_1 = 20.1 mM^{-1} s^{-1}, at 20 MHz and 298 K) was measured in the case of an analogous trinuclear system with three [Gd(DTTA)$(H_2O)_2$]$^-$ chelates grafted on the same central unit.[7] The remarkable proton relaxivity of this latter complex was explained by the limited flexibility of the molecule, *i.e.* hindered internal rotation about the CH_2 linker between the benzene core and the Gd-complex.

The $1/T_1$ NMRD profile of Gd_3**L4** has a shape which reproduces that typical of medium-size multimeric complexes: a region of constant relaxivity at low frequencies (ca. 0.01–2 MHz), a dispersion around 2–6 MHz and then a broad hump at high fields, with a maximum around 60–80 MHz (Figure 4).[2] The analysis of the data was performed as described above, using the same values for the rate of exchange of coordinated water molecules as those measured for the mononuclear complex. The justification for this choice comes from the measurement of the temperature dependence of r_1 (20 MHz) which indicates also for the trinuclear complex the occurrence of the fast exchange condition (Figure S1). Unlike the mononuclear complexes, the rotational dynamics has been analysed by using the Lipari-Szabo approach that allows the separate assessment of the contributions of the local motion of the Gd-chelates, described by a local rotational correlation time, τ_{RL}, and the overall motion of the trinuclear system, characterized by a global rotational correlation time, τ_{RG}.[26] The degree of correlation between the two types of motions is given by the parameter S^2, which can assume values between zero (completely independent motions) and one (totally correlated motions). The experimental NMRD curve is accurately reproduced with the parameters listed in Table 1, which clearly indicate the occurrence of a degree of local flexibility not entirely negligible, evidenced by the τ_{RL} and τ_{RG} values that differ by about a factor of three. Thus, even in the case of this relatively compact trinuclear complex rotational dynamics dominates the relaxivity at high fields. Larger r_1 values can only be obtained by increasing the degree of correlation of the two types of motion, thus limiting the ease of rotation of the complex around the linker.

The NMRD profile of Gd_3**L3** differs markedly from that of Gd_3**L4** since the r_1 values show a very limited dependence on the proton Larmor frequency (Figure 4). In addition, in the magnetic field region relevant to MRI (> 20 MHz), the Gd_3**L3** relaxivity is significantly lower than that of Gd_3**L4**. In the formation of the trinuclear complex, the carboxylic acid of the hexanoic chain is involved in conjugation to the central macrocyclic unit and therefore unavailable for coordination to the Gd^{III} ion. Therefore, in the trinuclear we expect a hydration state of two (q = 2) for the Gd^{3+} ion. Instead, the shape and amplitude of the NMRD profile are compatible with both a lower q value and a greater flexibility of the chelates in the trinuclear system. A plausible hypothesis, in agreement with the occurrence of both factors, is the coordination of a carbonyl group bound to the central macrocyclic to one Gd^{3+} center, resulting in the displacement of one water molecule. In

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this case, the trinuclear complex would consist of two chelates in which the Gd ion has q = 2 and a unit with q = 1. The effective q value would therefore be 1.67. The experimental data were analyzed by fixing q to this value, and using the same procedure described in the case of Gd_3**L4**. The relaxation parameters, listed in Table 1, support the above hypothesis. In fact, unlike Gd_3**L4**, we notice a much more marked difference between local and global motion, as evidenced by the low value of the S^2 parameter. This reflects the ease of rotation of the two chelates with q = 2 around the long alkyl chain. The remarkable flexibility of the system results in a clear limitation of relaxivity at high fields and reduced frequency dependence. The greater change of Δ^2 from Gd**L1** to Gd_3**L3** could instead be a consequence of the remarkable difference of the coordination cage of Gd^{3+} in the two systems.

Experimental

All chemicals were purchased from Sigma-Aldrich or Alfa Aesar unless otherwise stated and were used without further purification. The ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Advance III 500 MHz (11.4 T) spectrometer equipped with 5mm PABBO probes and BVT-3000 temperature control unit. Chemical shifts are reported relative to TMS and were referenced using the residual proton solvent resonances. HPLC purifications and mass spectra were performed on a Waters HPLC-MS system equipped with a Waters 1525 binary pumps. A Waters Atlantis prep T3 OBD (5µm 19x100mm) was used for preparative purposes. Electrospray ionization mass spectra (ESI MS) were recorded using a SQD 3100 Mass Detector (Waters), operating in positive or negative ion mode, with 1% v/v formic acid in methanol as the carrier solvent.

Synthesis of 1-(methylhexanoate)-4,7,10-tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane (1).

To a CH_3CN solution of 100 mg (0.19 mmol) of 4,7,10-tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane, 61 mg (0.29 mmol) of methyl-6-bromohexanoate, K_2CO_3 134 mg (0.97 mmol), and a catalytic amount of KI (6 mg, 0.04 mmol) were added and left stirring at r.t overnight. The reaction mixture was filtered, evaporated in vacuo, and purified by silica gel chromatography (from 99:1-> 90:10 CH_2Cl_2:MeOH) to afford **1** (74 mg, 0.11 mmol, yield 60%) ^{1}H NMR (500 MHz, $CDCl_3$): 1.43 (m, 2H, -NH$(CH_2)_2$*CH*$(CH_2)_2$CO-); 1.42 (s, 27H,-COO*(CH$_3$)$_3$*) 1.64 (m, 2H, -NCH_2*C*$H_2(CH_2)_3$CO-); 1.82 (m, 2H, -N$(CH_2)_3$*CH*$_2$CH_2CO-); 2.44 (m, 2H, -N$(CH_2)_4$*CH*$_2$CO-); 3.08 (2H, m, -N*CH*$_2(CH_2)_4$CO-); 3.10-3.56 (m, 16H, cyclen); 3.64 (s, 3H, -*OCH*$_3$); 3.96 (s, 6H, -N*CH*$_2$COO*(CH$_3$)$_3$*). ^{13}C NMR (125 MHz, $CDCl_3$): 22.1 (-N$(CH_2)_3$*CH*$_2$CH_2CO-); 24.0 (-NH$(CH_2)_2$*CH*$(CH_2)_2$CO-); 25.1 (-NCH_2*CH*$_2$(CH2)$_3$CO-); 28.7 (-COO*C(CH$_3$)$_3$*); 32.7 (-N$(CH_2)_4$*CH*$_2$CO-); 47.9-54.2 (cyclen); 51.24 (-OCH_3); 55.8 (-N*CH*$_2$COO*C(CH$_3$)$_3$*); 56.4 (-N*CH*$_2(CH_2)_4$CO-); 83.3 (-NCH_2COO*C*$(CH_3)_3$), 169.5 ((-NCH_2*CO*O$C(CH_3)_3$)); 174.3 (-N$(CH_2)_5$*CO*-) ESI-MS (m/z): found 643.17 (M+H$^+$) (calc for $C_{33}H_{62}N_4O_8$: 643.88).

Synthesis of 1-(hexanoic)-4,7,10-tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane (2)

Compound **1** (74 mg, 0.11 mmol) was dissolved in 3 ml MeOH: H_2O (1:1) and LiOH (10 mg, 0.42 mmol) was added and the mixture was stirred at RT overnight. The mixture was concentrated in vacuo to remove methanol and then lyophilized to afford **2** (70 mg, 98% yield). ^{1}H NMR (500 MHz, $CDCl_3$): 1.43 (m, 2H, -NH$(CH_2)_2$*CH*$(CH_2)_2$CO-); 1.40 (s, 27H ,-COO*(CH$_3$)$_3$*) 1.65 (m, 2H, -NCH_2*C*$H_2(CH_2)_3$CO-); 1.84 (m, 2H, -N$(CH_2)_3$*CH*$_2$CH_2CO-); 2.41 (m, 2H, -N$(CH_2)_4$*CH*$_2$CO-); 3.09 (2H, m, -N*CH*$_2(CH_2)_4$CO-); 3.11-3.60 (m, 16H, cyclen); 3.92 (s, 6H, -N*CH*$_2$COO$(CH_3)_3$). ^{13}C NMR (125 MHz, $CDCl_3$): 22.5 (-N$(CH_2)_3$*CH*$_2$CH_2CO-); 24.5 (-NH$(CH_2)_2$*CH*$(CH_2)_2$CO-); 25.8 (-NCH_2*CH*$_2(CH_2)_3$CO-); 32.7 (-N$(CH_2)_4$*CH*$_2$CO-); 47.7-54.1 (cyclen); 54.5 N*CH*$_2(CH_2)_4$CO-); 56.21 (-N*CH*$_2$COO$(CH_3)_3$); 81.6 (-NCH_2COO*C*$(CH_3)_3$), 169.2 ((-NCH_2*CO*O$C(CH_3)_3$)); 174.8 (-N$(CH_2)_5$*CO*-) ESI-MS (m/z): found 629.17 (M+H$^+$) (calc for $C_{32}H_{60}N_4O_8$: 629.88).

Synthesis of 1-(hexanoic)-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic acid (L1)

Compound **2** (70 mg, 0.11 mmol) was dissolved in 3 ml DCM: TFA (1:1) and stirred at RT overnight. The mixture was concentrated in vacuo and then lyophilized to afford **L1** in quantitative yield. ^{1}H NMR (500 MHz, D_2O): 1.43 (t, 2H, J = 7.6 Hz, -NH$(CH_2)_2$*CH*$_2(CH_2)_2$CO-); 1.68 (t, J = 7.6 Hz, 2H, -NCH_2*CH*$_2(CH_2)_3$CO-); 1.81 (m, 2H, -N$(CH_2)_3$*CH*$_2$CH_2CO-); 2.44 (t, 2H, J = 7.6 Hz -N$(CH_2)_4$*CH*$_2$CO-); 3.09-3.60 (m, 22H, cyclen and -N*CH*$_2$COOH); 3.99 (s, 2H, -N*CH*$_2(CH_2)_4$CO). ^{13}C NMR (125 MHz, $CDCl_3$): 22.0 (-N$(CH_2)_3$*CH*$_2$CH_2CO-); 23.0 (-NH$(CH_2)_2$*CH*$(CH_2)_2$CO-); 24.6 (-NCH_2*CH*$_2$(CH2)$_3$CO-); 32.7 (-N$(CH_2)_4$*CH*$_2$CO-); 47.5, 48.0, 49.3, 51.6 (cyclen); 52.3 (-N*CH*$_2(CH_2)_4$CO-); 54.1 (-N*CH*$_2$COOH); 173.7 ((-NCH_2*CO*OH); 177.8 (-N$(CH_2)_5$*CO*-) ESI-MS (m/z): found 461.67 (M+H$^+$) (calc for $C_{20}H_{36}N_4O_8$: 461.24).

Synthesis of the *t*Bu protected hexanoate-DO3A-trimer (3)

Compound **2** (70 mg, 0.11 mmol) was dissolved in DMF (2 mL) with 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU; 0.035 g, 0.092 mmol) in presence of triethylamine (26 µL,0.18 mmol) and stirred for 2 min before adding a solution of 1,4,7-triazacyclononane (3 mg, 0.023 mmol). After stirring overnight, the reaction mixture was concentrated in vacuo and purified by silica gel chromatography (90:10 CH_2Cl_2:MeOH) to obtain compound **3** (25 mg, 0.012 mmol, 55%). ^{1}H NMR (500 MHz, $CDCl_3$): 1.42 (m, 29H, -COO*C(CH$_3$)$_3$*, -NH$(CH_2)_2$*CH*$(CH_2)_2$CO-); 1.65 (m, 2H, -NCH_2*CH*$_2$(CH2)$_3$CO-); 1.75 (m, 2H, -N$(CH_2)_3$*CH*$_2$CH_2CO-); 2.41 (m, 2H, -N$(CH_2)_4$C*H*$_2$CO-); 2.99-3.73 (m, 28H, from both macrocycles); 3.89 (2H, m, -NC*H*$_2(CH_2)_4$CO-); 4.08 (s, 6H, -N*CH*$_2$COO$(CH_3)_3$). ^{13}C NMR (125 MHz, $CDCl_3$): 22.6 (-N$(CH_2)_3$*CH*$_2$CH_2CO-); 24.4 (-NH$(CH_2)_2$*CH*$(CH_2)_2$CO-); 25.7 (-NCH_2*CH*$_2(CH_2)_3$CO-); 28.2 (-COO*C(CH$_3$)$_3$*) 32.86 (-N$(CH_2)_4$*CH*$_2$CO-); 47.80-53.6 (macrocycles); 54.3 N*CH*$_2(CH_2)_4$CO-); 56.2 (-N*CH*$_2$COO$(CH_3)_3$); 81.9 (-NCH_2COO*C*$(CH_3)_3$), 169.4 ((-NCH_2*CO*O$C(CH_3)_3$); 174.6 (-N$(CH_2)_5$*CO*-) ESI-MS (m/z): found 1961.42 (M+H$^+$) (calc for $C_{102}H_{189}N_{15}O_{21}$: 1961.62).

Synthesis of the deprotected hexanoate-DO3A-trimer (L3)

Compound **3** (25 mg, 0.012 mmol) was dissolved in a solution of DCM:TFA 1:1 (5 mL) and was left stirring overnight, the reaction mixture was concentrated in vacuo to give **L1** (15 mg,

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0.011 mmol, 90%). ^{1}H NMR (500 MHz, $CDCl_3$): 1.41 (m, 2H, -NH(CH_2)$_2$*CH*(CH_2)$_2$CO-); 1.63 (m, 2H, -NCH$_2$*CH*$_2$(CH_2)$_3$CO-); 1.74 (m, 2H, -N(CH_2)$_3$*CH*$_2$$CH_2$CO-); 2.40 (m, 2H, -N($CH_2$)$_4$*CH*$_2$CO-); 2.92-3.70 (m, 28H, macrocycles); 3.83 (2H, m, -N*CH*$_2$(CH_2)$_4$CO-); 4.01 (s, 6H, -N*CH*$_2$COOH). ^{13}C NMR (125 MHz, $CDCl_3$): 22.7 (-N(CH_2)$_3$*CH*$_2$$CH_2$CO-); 24.3 (-NH($CH_2$)$_2$*CH*($CH_2$)$_2$CO-); 25.6 (-NCH$_2$*CH*$_2$(CH2)$_3$CO-); 32.7 (-N($CH_2$)$_4$$CH_2$CO-); 47.9-53.2 (both macrocycles); 54.2 (-N*CH*$_2$(CH_2)$_4$CO-); 56.1 (-N*CH*$_2$COOH); 169.7 ((-NCH$_2$*CO*OH)); 174.3 (-N(CH_2)$_5$*CO*-) ESI-MS (m/z): found 1457.52 (M+H$^+$) (calc for $C_{66}H_{117}N_{15}O_{21}$: 1457.72).

Synthesis of methyl-4-methylbenzoate-4,7,10-tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane (4)

To a CH_3CN solution of 100 mg (0.19 mmol) of 4,7,10-tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane, 66 mg (0.29 mmol) of methyl 4-(bromomethyl)benzoate, K_2CO_3 134 mg, 0.97 mmol) and a catalytic amount of KI (6 mg, 0.04 mmol) were added and left stirring at rt overnight. The reaction mixture was filtered, evaporated in vacuo and purified by silica gel chromatography (from 99:1-> 90:10 CH_2Cl_2: MeOH) to afford **4** (68 mg, 0.10 mmol, yield 54%) ^{1}H NMR (500 MHz, $CDCl_3$): 1.40 (s, 27H, -COOC*(CH$_3$)$_3$*) 3.10-3.56 (m, 16H, cyclen); 3.66 (s, 2H, -N*CH*$_2$C$_6$H$_4$COOCH$_3$) 3.72 (s, 6H, -N*CH*$_2$COOC(CH$_3$)$_3$); 3.78 (s, 3H, -*OCH*$_3$*)*; 7.45 (d, *J* = 8.1 Hz, 2H, Ph$_{ortho}$), 7.86 (d, *J* = 8.1 Hz, 2H, Ph$_{meta}$). ^{13}C NMR (125 MHz, $CDCl_3$): 27.7 (-COOC*(CH$_3$)$_3$*); 47.9-54.2 (cyclen); 51.5 (-*OCH*$_3$); 55.10 (-N*CH*$_2$COOC(CH$_3$)$_3$); 60.4 (-N*CH*$_2$C$_6$H$_4$COOCH$_3$); 82.3 (-NCH$_2$COO*C*(CH$_3$)$_3$); 128.7 (-CH$_2$, Ph$_{ortho}$); 129 (C); 129.6 (-CH$_2$, Ph$_{meta}$); 142.9 (C); 164.47 ((-NCH$_2$C$_6$H$_4$*CO*OCH$_3$); 173.12 (-NCH$_2$*CO*OC(CH$_3$)$_3$); ESI-MS (m/z): found 663.57 (M+H$^+$) (calc for $C_{35}H_{58}N_4O_8$: 662.87.

Synthesis of 4-methylbenzoic-4,7,10-tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane (5)

Compound **4** (68 mg, 0.10 mmol) was dissolved in 3 ml MeOH: H_2O (1:1) and LiOH (9 mg, 0.4 mmol) was added and stirred at RT overnight. The mixture was concentrated in vacuo to remove methanol and then lyophilized to afford **5** (62 mg, 96% yield). ^{1}H NMR (500 MHz, $CDCl_3$): 1.44 (s, 27H, -COOC*(CH$_3$)$_3$*) 3.10-3.56 (m, 16H, cyclen); 3.66 (s, 2H, -N*CH*$_2$C$_6$H$_4$COOH) 3.72 (s, 6H, -N*CH*$_2$COOC(CH$_3$)$_3$); 7.27 (d, *J* = 8.1 Hz, 2H, Ph$_{ortho}$), 7.96 (d, 2H, *J* = 8.1 Hz, Ph$_{meta}$). ^{13}C NMR (125 MHz, $CDCl_3$): 27.9 (-COO*(CH$_3$)$_3$*); 47.9-54.2 (cyclen); 55.8 (-N*CH*$_2$COOC(CH$_3$)$_3$); 59.2 (-N*CH*$_2$C$_6$H$_4$COOH); 82.8 (-NCH$_2$COO*C*(CH$_3$)$_3$); 129.2 (-CH$_2$, Ph$_{ortho}$); 129.7 (C); 129.9 (-CH$_2$, Ph$_{meta}$); 142.9 (C); 166.3 ((-NCH$_2$C$_6$H$_4$*CO*OH); 173.2 (-NCH$_2$*CO*OC(CH$_3$)$_3$); ESI-MS (m/z): found 649.64 (M+H$^+$) (calc for $C_{34}H_{56}N_4O_8$: 649.84).

Synthesis of 1-(4-methylbenzoic)-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic acid (L2)

Compound **5** (69 mg, 0.10 mmol) was dissolved in 3 ml DCM:TFA (1:1) and stirred at RT overnight. The mixture was concentrated in vacuo and then lyophilized to afford **L2** in quantitative yield. ^{1}H NMR (500 MHz, D2O): 3.03-3.56 (m, 22H, cyclen and -N*CH*$_2$COOH); 4.06 (s, 2H, -N*CH*$_2$(CH_2)$_4$CO), 7.52 (d, *J* = 8.1 Hz, 2H, Ph$_{ortho}$), 7.84 (d, 2H, *J* = 8.1 Hz, Ph$_{meta}$). ^{13}C NMR (125 MHz, $CDCl_3$): 48.5, 49.5, 49.7, 50.3 (cyclen); 56.0 N*CH*$_2$(CH_2)$_4$CO-); 57.1 (-N*CH*$_2$COOH); 129.4 (-CH$_2$, Ph$_{ortho}$); 129.6 (C); 130.9 (-CH$_2$, Ph$_{meta}$); 136.9 (C); 173.0 (-NCH$_2$*CO*OH), 175.3 ((-NCH$_2$C$_6$H$_4$*CO*OH); ESI-MS (m/z): found 481.64 (M+H$^+$) (calc for $C_{34}H_{56}N_4O_8$: 481.84).

Synthesis of the *t*Bu protected benzoate-DO3A-trimer (6)

Compound **5** (62 mg, 0.09 mmol) was dissolved in DMF (2 mL) with 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU; 41 mg , 0.1 mmol) in presence of triethylamine (26 μL,0.18 mmol) and stirred for 2 min before adding a solution of 1,4,7-triazacyclononane (2.5 mg, 0.018 mmol). After stirring overnight, the reaction mixture was concentrated in vacuo and purufied by silica gel chromatography (90:10 CH_2Cl_2:MeOH) to obtain compound **6** (15 mg, 0.0072 mmol, 40%). ^{1}H NMR (500 MHz, $CDCl_3$): 1.44 (s, 27H, -COOC*(CH$_3$)$_3$*) 3.10-3.62 (m, 28H, from both macrocycles); 3.66 (s, 2H, -N*CH*$_2$C$_6$H$_4$CO-) 3.72 (s, 6H, -N*CH*$_2$COOC(CH$_3$)$_3$); 7.39 (d, *J* = 8.1 Hz, 2H, Ph$_{ortho}$), 7.99 (d, *J* = 8.1 Hz, 2H, Ph$_{meta}$). ^{13}C NMR (125 MHz, $CDCl_3$): 28.1 (-COOC*(CH$_3$)$_3$*); 47.7-55.7 (from both macrocycles); 56.7 (-N*CH*$_2$COOC(CH$_3$)$_3$); 56.8 (-N*CH*$_2$C$_6$H$_4$CO-); 81.8 (-NCH$_2$COO*C*(CH$_3$)$_3$); 128.7 (-CH$_2$, Ph$_{ortho}$); 129.6 (-CH$_2$, Ph$_{meta}$); 133.7 (C); 139.9 (C); 168.5 ((-NCH$_2$C$_6$H$_4$*CO*-); 169.2 (-NCH$_2$*CO*OC(CH$_3$)$_3$); ESI-MS (m/z): found 2022.62 (M+H$^+$), calc for $C_{108}H_{177}N_{15}O_{21}$: 2021.69).

Synthesis of the deprotected benzoate-DO3A-trimer (L4)

Compound **6** (15 mg, 0.0072 mmol) was dissolved in a solution of DCM:TFA 1:1 (5 mL) and was left stirring overnight, the reaction mixture was concentrated in vacuo to give **L2** (11 mg, 0.064 mmol, 88%). ^{1}H NMR (500 MHz, $CDCl_3$): 3.10-3.62 (m, 28H, from both macrocycles); 3.66 (s, 2H, -N*CH*$_2$C$_6$H$_4$CO-) 3.72 (s, 6H, -N*CH*$_2$COOH); 7.52 (d, *J* = 8.1 Hz, 2H, Ph$_{ortho}$), 7.84 (d, *J* = 8.1 Hz, 2H, Ph$_{meta}$). ^{13}C NMR (125 MHz, $CDCl_3$): 48.5-56.1 (from both macrocycles); 56.7 (-N*CH*$_2$COOH); 57.1 (-N*CH*$_2$C$_6$H$_4$CO-); 12.5 (-CH$_2$, Ph$_{ortho}$); 129.6 (-CH$_2$, Ph$_{meta}$); 130.9 (C); 136.9 (C); 173.0 ((-NCH$_2$C$_6$H$_4$*CO*-); 175.3 (-NCH$_2$*CO*OH); ESI-MS (m/z): found 1517.52 (M+H$^+$) (calc for $C_{72}H_{105}N_{15}O_{21}$: 1516.72).

Relaxometric measurements.

The water proton longitudinal relaxation rates as a function of the magnetic field strength were measured in non-deuterated aqueous solutions on a Fast Field-Cycling Stelar SmarTracer relaxometer (Stelar s.r.l., Mede (PV), Italy) over a continuum of magnetic field strengths from 0.00024 to 0.25 T (corresponding to 0.01–10 MHz proton Larmor frequencies). The relaxometer operates under computer control with an absolute uncertainty in $1/T_1$ of ±1%. Additional longitudinal relaxation data in the range 15–70 MHz were obtained on a Stelar Relaxometer connected to a Bruker WP80 NMR electromagnet adapted to variable-field measurements. The exact concentration of Gd(III) was determined by measurement of bulk magnetic susceptibility shifts of a *t*BuOH signal or by inductively coupled plasma mass spectrometry (ICP-MS, Element-2, Thermo-Finnigan, Rodano (MI), Italy). Sample digestion was performed with concentrated HNO_3 (70%, 2 mL) under microwave heating at 160°C for 20 min (Milestone MicroSYNTH Microwave lab station equipped with an optical fibre temperature control and HPR-1000/6 M six position high pressure reactor, Bergamo, Italy). The ^{1}H T_1 relaxation times were acquired by the standard inversion

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recovery method with typical 90° pulse width of 3.5 μs, 16 experiments of 4 scans. The temperature was controlled with a Stelar VTC-91 airflow heater equipped with a calibrated copper-constantan thermocouple (uncertainty of ±0.1 °C).

Variable-temperature ^{17}O NMR measurements were recorded on a Bruker Avance III spectrometer (11.7 T) equipped with a 5 mm probe and standard temperature control unit. Aqueous solutions of the complexes containing 2.0% of the ^{17}O isotope (Cambridge Isotope) were used. The observed transverse relaxation rates were calculated from the signal width at half-height.

Conclusions

Bishydrated GdDO3A functionalized derivatives bearing a suitable substituent on the secondary nitrogen of the macrocyclic ring have been synthesized and conjugated to a central unit, resulting in trinuclear complexes. The nature of the pendant group has a significant influence on the relaxometric properties of both mononuclear and multinuclear Gd^{III} complexes. The length and flexibility of the pendant chain and the presence of a good donor group result in the perturbation of the inner coordination sphere of the Gd^{3+} ion, whose hydration state decreases from two to one in Gd**L1**. Interestingly, given the relatively low stability of the nine-membered Gd-N-$(CH_2)_5$-CO-Gd ring, the carbonate anion competes for the coordination of Gd^{III} and, between pH ca. 8-10, an equilibrium exists between species $q = 1$ and $q = 0$. The greater rigidity of the pendant group in **L2** prevents this process in Gd**L2** that, therefore, maintains the expected features of a bishydrated complex. The chemical nature and electronic effects of the pendant group mainly affect the rate of water exchange, which is significantly accelerated compared to that measured for the parent GdDO3A complex.

The length, flexibility and steric hindrance of the linker connecting the chelates to the central unit affect the rotational dynamics of the trinuclear complexes, hence their relaxometric properties, particularly at the fields relevant for MRI. In Gd_3**L3,** the flexibility of the alkyl chain has two significant consequences: a) displacement of a water molecule on one Gd-complex by a carbonyl group of the central unit; b) easy local rotation about the chain that results in a marked difference between local and global motion. Both of these factors contribute to limiting the relaxivity and thus the effectiveness of the trinuclear complex. In Gd_3**L4**, the lower rotational flexibility entails a better motional coupling between local and overall tumbling motion and consequently enables a high relaxivity, approximately three times higher (per Gd) than that of Gd-based MRI contrast agents clinically used.

Conflicts of interest

There are no conflicts to declare.

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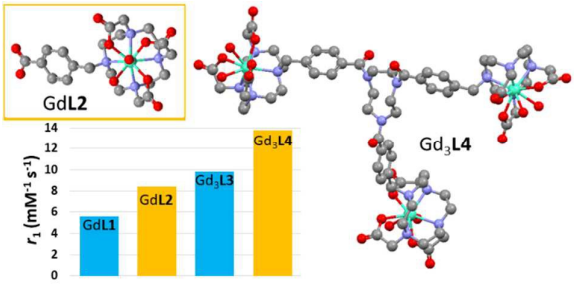

The flexibility/rigidity of the linker causes different relaxometric behavior on both mononuclear and trinuclear Gd^{III} complexes based on DO3A-like structures.