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Multinuclear and multifrequency NMR study of gadolinium(III) complexes with bis-amide derivatives of ethylenedioxydiethylenedinitrilotetraacetic acid

Silvio Aime,*[a] Alessandro Barge,[a] Mauro Botta,[b] Luca Frullano,[a] Ugo Merlo[a] and Kenneth I. Hardcastle[c]

[a] *Dipartimento di Chimica I.F.M., Universita' di Torino, Via P. Giuria 7, I-10125 Torino, Italy*
[b] *DISTA, Universita' del Piemonte Orientale "Amedeo Avogadro", Corso Borsalino 54, I-15100 Alessandria, Italy*
[c] *Department of Chemistry, Emory University, 1515 Pierce Drive, Atlanta 30322, GA, USA*

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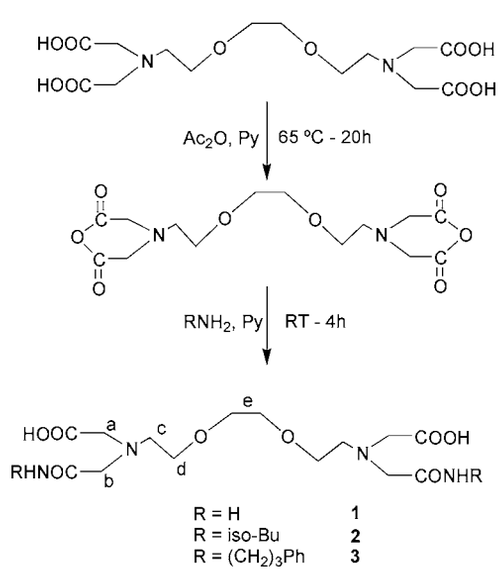
New cationic gadolinium complexes have been synthesized and their ^{1}H and ^{17}O relaxometric properties investigated in view of their possible use as MRI contrast agents. The reaction of the bicyclic anhydride of ethylenedioxydiethylenedinitrilotetraacetic acid (H_4egta) with NH_3, isobutylamine and phenylpropylamine gave the octadentate bis-amide ligands **1**–**3** in high yields. The gadolinium(III) complexes were prepared from $GdCl_3$ and the acid form of the ligands. The stability constants of the complexes were obtained from potentiometric data and are about five orders of magnitude lower than for the parent $[Gd(egta)]^-$ complex. The magnetic field dependence of the proton relaxivity at 6, 25 and 39 °C was quantitatively analysed in order to obtain the relaxation parameters and indicated that one water molecule occupies a site of the co-ordination sphere of the complexes. The temperature dependence of the relaxivity, measured at 20 MHz, suggested that the water exchange rate, $k_{ex} = 1/\tau_M$, is sensibly reduced compared to that of the parent compound. ^{17}O NMR data were acquired at 2.1 T and allowed an accurate estimation of τ_M which, at 298 K, resulted to be 230, 133 and 211 ns for Gd·**1**, Gd·**2** and Gd·**3** respectively. The two complexes bearing hydrophobic residues on the amide nitrogens form non-covalent adducts with serum albumin whose relaxivity is limited by the water exchange rate.

Introduction

The monoaqua, ennea-co-ordinated gadolinium(III) complexes currently in use as Contrast Agents (CAs) for Magnetic Resonance Imaging (MRI) are characterized by a slow exchange rate ($k_{ex} = 1/\tau_M$) of the inner co-ordination sphere water molecule(s).[1] This fact limits the relaxivity (r_{1p}), the paramagnetic relaxation rate of the solvent per mmol L^{-1} concentration of the CA, in principle attainable for the supramolecular adducts with slowly tumbling substrates, since the transfer of the paramagnetic effects to the solvent, modulated by the exchange of the metal bound water molecule(s), is not optimized. The paramagnetic contribution of this relaxation mechanism (*inner sphere*) to the observed water proton longitudinal relaxation rate is given by eqn. (1) where M is the molar concentration of the

$$R_{1p}^{is} = \frac{[M]q}{55.6}\frac{1}{T_{1M} + \tau_M} \qquad (1)$$

gadolinium(III) complex, q the number of metal bound water molecules and T_{1M} their longitudinal relaxation time.[2] When the complex is immobilized by conjugation to a macromolecule T_{1M} becomes very short and if $\tau_M \geq T_{1M}$ (slow exchange condition) then the relaxivity is limited by the value of the mean residence lifetime τ_M. Whereas an ideal value of τ_M can be calculated to be about 30 ns at 25 °C, both $[Gd(dota)]^-$ (H_4dota = 1,4,7,10-tetrakis(carboxymethyl)-1,4,7,10-tetraazacyclododecane) and $[Gd(dtpa)]^{2-}$ [H_5dtpa = carboxymethyliminobis(ethylenenitrilo)tetraacetic acid], the two reference compounds that first became available for clinical use, have values for this parameter of about 300 ns.[3] The value further increases by an order of magnitude for the neutral derivatives when one (dota) or two (dtpa) carboxylate groups of the ligand are replaced by hydroxypropyl or carboxamide moieties. We have recently found that $[Gd(egta)]^-$ (H_4egta = ethylenedioxydiethylenedinitrilotetraacetic acid) shows a remarkably fast solvent exchange rate which has been explained in terms of the solution structure of the complex featuring severe constraints at the water binding site attributed to the oxoethylenic bridge.[4] In the search for a better understanding of the relationship between the exchange rate of the co-ordinated water, the geometry at the Gd^{III} and the overall electric charge of the complex, we have synthesized and characterized by multinuclear and multifrequency NMR a series of gadolinium(III) complexes with bisamide derivatives of egta (Scheme 1). Two



Scheme 1

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Table 1 Potentiometric protonation and stability constants[a]

Equilibrium	log *K*						
	edta[b]	**dtpa**[b]	**dtpa-bma**[c]	**egta**[b]	**1**	**2**	**3**
$HL \rightleftharpoons H + L$	10.17	10.55	9.37	9.47	6.79	6.55	6.58
$H_2L \rightleftharpoons H + HL$	6.11	8.59	4.38	8.85	5.87	5.36	5.21
$H_3L \rightleftharpoons H + H_2L$	2.68	4.30	3.31	2.26	2.79	3.30	2.06
$H_4L \rightleftharpoons H + H_3L$	2.0	2.66	1.43	2.00			
$H_5L \rightleftharpoons H + H_4L$	1.5	1.82					
$GdL \rightleftharpoons Gd + L$	17.35	22.46	16.86	17.50		11.84	11.08
$Gd(HL) \rightleftharpoons GdL + H$						8.20	2.81
$Gd(H_2L) \rightleftharpoons Gd(HL) + H$						4.35	

[a] At 25 °C, $I = 0.1$ mol L^{-1} (KCl). [b] From ref. 6. [c] From ref. 5.

of the monocationic complexes have the amide nitrogens substituted with an aromatic group or an aliphatic chain and thus are able non-covalently to bind slowly tumbling macromolecular substrates such as bovine serum albumin (BSA). The formation of these adducts and their relaxometric properties have also been investigated.

Results and discussion

Synthesis

The ligands **1**–**3** were prepared by a two step procedure. First, H_4egta was treated with acetic anhydride in pyridine to give the bis(anhydride) derivative which was isolated as a brown oil after removal of the solvent under reduced pressure. Then the egta bis(anhydride) was treated with an amine RNH_2 in pyridine to give the corresponding ligand that was purified by removing the excess of amine by extraction into water at pH 10 using diethyl ether. The lanthanide complexes were prepared from the corresponding chlorides.

Protonation constants

The macroscopic protonation constants for the ligands **1**–**3** were calculated from potentiometric titration curves; their values are reported in Table 1 which also contains the values for related linear polyamino polycarboxylic ligands, for comparison. The substitution of two carboxylates with carboxamide groups decreases the value of the first two protonation constants by an order of magnitude in the case of the *N*,*N*″-bismethylamide derivative of dtpa,[5] *i.e* dtpa-bma, an analogous substitution results in constant values for the ligands **1**–**3** about three orders of magnitude lower than for egta.[6] This effect is likely to be associated with the lower negative charge of free **1**–**3**.

Information on the microscopic protonation sequence can be deduced from the ^{1}H NMR titration curves obtained by measuring the changes of the chemical shift values for the non-labile protons of the ligand as a function of the pD. Generally, the protonation of a basic site of a ligand induces a deshielding of the adjacent non-labile protons. This allows the assignment of each macroscopic protonation constant to the appropriate equilibrium.[7,8] As an example, in Fig. 1 are reported the ^{1}H NMR titration curves for selected protons of ligand **1**. Around pD 6.5 the most affected resonances are those corresponding to protons a–c which indicates that the first two protonation steps involve the two amino nitrogen atoms. At pD lower than 3 only protons b are significantly affected since the third protonation occurs on a carboxyl group.

Stability constants of the gadolinium(III) complexes

From potentiometric data the stability constants, log K_{ML}, for the gadolinium complexes with ligands **2** and **3** were calculated to be 11.8 and 11.1, respectively. In analogy with the case of dtpa, substitution of two carboxylates with carboxamide moieties decreases the log *K* values by about 5 orders of magnitude.[5,6] Whereas for dtpa-bma the reduced stability of the gadolinium(III) complex is counterbalanced by the higher specificity of the ligand for Gd relative to other transition metal ions, thus resulting in a low toxicity of the CA, in our case the stability constants appear too low for a possible *in vivo* utilization.

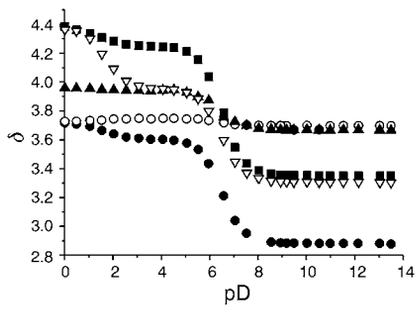

Fig. 1 pD Dependence of the ^{1}H NMR chemical shifts (400 MHz) of ligand **1**. Resonances: a (filled squares), b (down triangles), c (filled circles), d (up triangles) and e (open circles).

Relaxation properties

The relaxivity describes the efficiency of magnetic dipolar coupling occurring between the solvent nuclei and the paramagnetic metal ion and represents a measure of the efficacy of the complex as a CA. The electron–nuclear interaction is generally described as the sum of two contributions involving the water molecules present in the inner co-ordination sphere of the metal ion and those randomly diffusing next to the complex (*outer sphere* mechanism). A complete discussion of the theory of paramagnetic relaxation with all the relevant equations has been given earlier.[2,3,9,10] Here we only give some general information and introduce the parameters useful for the analysis of the data. The *inner sphere* proton relaxation mechanism depends on the number of co-ordinated water molecules, *q*, their mean residence lifetime, τ_M, the distance between the water protons and the metal center, *r*, the electronic relaxation time of the gadolinium ion, τ_S, and the reorientational correlation time of the whole complex, τ_R. The electronic relaxation time is dependent on the magnetic field strength and is given in terms of a correlation time, τ_V, characterizing the time fluctuations of the electron spin–lattice interaction and its zero-field value, τ_{S0}. The *outer sphere* relaxivity depends upon τ_S, the relative diffusion coefficent of solute and solvent, *D*, and the distance of minimum approach between the metal ion and the protons of the diffusing water molecules, *a*. Finally, r_{1p} has a strong dependence on the applied magnetic field strength, described by the Solomon–Bloembergen–Morgan (*inner sphere*) and Freed (*outer sphere*) equations. When the residence lifetime is not a limiting factor, the value of r_{1p} at high magnetic fields (corresponding to proton Larmor frequencies >10 MHz) is primarily controlled by the value of τ_R which is strictly correlated with the molecular weight for complexes with the same hydration number, rigid and nearly spherical.

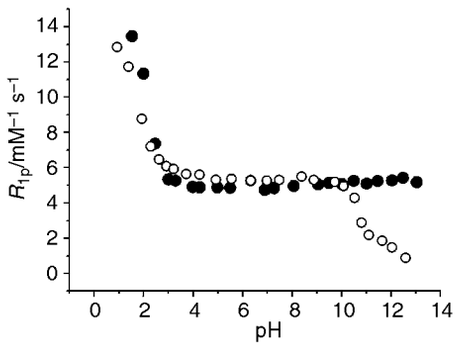

Fig. 2 pH Dependence of the longitudinal proton relaxivity of Gd·**1** (open circles) and $[Gd(egta)]^-$ (filled circles) at 20 MHz and 25 °C.

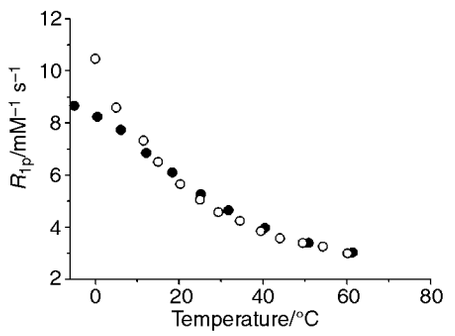

Fig. 3 Temperature dependence of the longitudinal proton relaxivity of Gd·**3** (filled circles) and $[Gd(egta)]^-$ (open circles) at 20 MHz and pH 7.2.

The relaxivity of the three complexes, as measured at 20 MHz and 25 °C, is 4.6, 5.6 and 5.3 mmol L^{-1} s^{-1} for Gd·**1**, Gd·**2** and Gd·**3**, respectively. These are values comparable to those of other complexes of similar size and $q = 1$, indicating that they all have the same hydration and similar τ_R values.[10] So we may assume that the gadolinium(III) complexes with **1**–**3** have one exchangeable water molecule and therefore that both carboxamide functionalities are involved in co-ordination. The pH dependence of the relaxivity was measured at 20 MHz and 25 °C for all the complexes and showed a strictly analogous behavior. As an example, the profile of Gd·**1** is shown in Fig. 2 together with that of $[Gd(egta)]^-$. The relaxivity is constant from pH 3 to 10, meaning that in this range the complexes do not exhibit changes in their hydration number, solution structure nor does partial dissociation occur. At pH below 3 the protonation of the carboxylate groups induces a relatively fast decomplexation and a steep increase of the relaxivity. Unlike the case of $[Gd(egta)]^-$, between pH 10 and 13 r_{1p} decreases to about 1 mmol L^{-1} s^{-1} and is accompanied by some precipitation. This could be the result of deprotonation of the co-ordinated water followed by formation of oxo-bridged insoluble polymeric species. In fact, unlike other cationic complexes where deprotonation of the bound water molecule can be observed in the relaxivity profile with pH,[11] the behavior of Gd·**1**–**3** is not fully reversible in the pH range 10–13 which indicates the occurrence of a significant structural change.

The relaxivity profile of complex Gd·**3** with temperature over the range 0–60 °C, at a pH of 7.2, is shown in Fig. 3 with the analogous profile for $[Gd(egta)]^-$. The relaxivity increases exponentially with lowering of the temperature as a result of the decrease of T_{1M} (eqn. 1) mainly as a consequence of the increase of the reorientational correlation time. However, below 10 °C the relaxivity deviates from the behavior of $[Gd(egta)]^-$ and flattens out. This is consistent with a changeover from the fast- to the slow-exchange condition and thus indicates that the water exchange rate is significantly lower for Gd·**3** than for the parent compound.

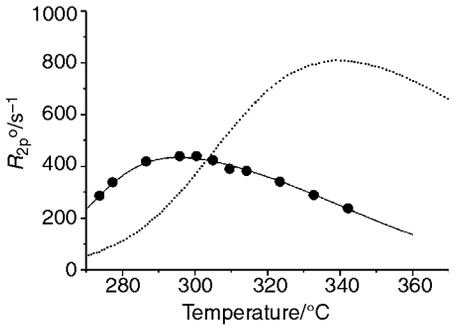

Fig. 4 Temperature dependence of the ^{17}O NMR transverse relaxation rate for Gd·**3** (●) and calculated profile of Gd(dtpa-bma) (dotted line; parameters taken from ref. 3) for 0.055 mol L^{-1} aqueous solutions at 12 MHz and pH 7.2.

Table 2 Parameters obtained from least-squares fits of ^{17}O NMR data

Complex	τ_M/ns	ΔH_M/ kJ mol^{-1}	τ_V/ns	ΔH_V/ kJ mol^{-1}	Δ^2/ 10^{19} s^{-2}
Gd·**1**	230	47.5	16	3.0	5.2
Gd·**2**	133	47.8	18	1.6	6.1
Gd·**3**	211	58.5	15	3.0	7.1
$[Gd(egta)]^-$ [a]	32	42.7	24		3.4
$[Gd(dtpa)]^{2-}$ [b]	303	51.6	25		4.6
Gd(dtpa-bma) [b]	2222	47.6	25		4.1

[a] From ref. 4. [b] From ref. 3.

Variable temperature ^{17}O NMR

An accurate determination of the exchange rate is possible by measuring the ^{17}O NMR transverse relaxation rate (R_{2p}°) as a function of temperature.[3,12] R_{2p}° is largely dominated by the scalar relaxation mechanism which is modulated by the electronic relaxation time τ_S [in terms of the parameters Δ^2 ($\Delta^2 = 1/12\ \tau_{So}\tau_V$) and τ_V] and by the exchange lifetime τ_M. By varying the temperature over a wide range it is possible, in most cases, to collect data in both the slow kinetic region at low temperature (R_{2p}° determined by $1/\tau_M$) and the fast kinetic region at high temperature (R_{2p}° determined by $1/\tau_S$) and thus obtain information on the activation parameters of τ_S (ΔH_V) and τ_M (ΔH_M). The experimental R_{2p}° profile with temperature for Gd·**3** is compared with the calculated profile of Gd(dtpa-bma) in Fig. 4.[7] The maximum in the profiles corresponds to the changeover from the slow to the fast kinetic regions. For Gd(dtpa-bma) a τ_M value of 2.2 μs at 25 °C was previously reported.[12] For Gd·**3** we may predict a shorter value since the maximum in the profile is shifted towards lower temperature. Very similar behavior is observed for Gd·**1** and Gd·**2**.

The obtained dynamic parameters for Gd·**1**–**3** are compared with the corresponding values for $[Gd(egta)]^-$, $[Gd(dtpa)]^{2-}$ and Gd(dtpa-bma) in Table 2. On passing from $[Gd(egta)]^-$ to the monocationic bis-amide derivatives the water exchange rate decreases by an order of magnitude and assumes a value very close to that of the anionic complexes $[Gd(dtpa)]^{2-}$ and $[Gd(dota)]^-$. A similar trend was observed on changing from $[Gd(dtpa)]^{2-}$ to Gd(dtpa-bma) and was interpreted as an effect of the weaker co-ordinating ability of the carboxamide groups that results in the ligand being less tightly pulled around the metal ion, thus partly releasing the steric constraint on the water binding site. The same effect is likely to be valid also for Gd·**1**–**3**; however, it is worth noting that the exchange lifetime for the positively charged egta bis-amide derivatives is about one order of magnitude lower than for the neutral complex Gd(dtpa-bma) as a result of the steric effect of the oxoethylenic bridge that favors destabilization of the bound water molecule. Furthermore, we have to consider that several isomers can be present in the aqueous solution of Gd·**1**–**3** differing in the relative position of the amide groups with respect to each other

Table 3 Best fit relaxation parameters determined by analysis of NMRD profiles

	Gd·**1**			Gd·**2**			Gd·**3**		
	6 °C	25 °C	39 °C	6 °C	25 °C	39 °C	6 °C	25 °C	39 °C
τ_{S0}/ps	66	85	72	78	89	71	76	87	69
τ_V/ps	16	12	8	22	19	11	21	19	9
τ_R/ps	110	68	52	117	85	71	124	88	73
r/Å	3.04	3.04	3.04	2.98	2.98	2.98	3.02	3.02	3.02

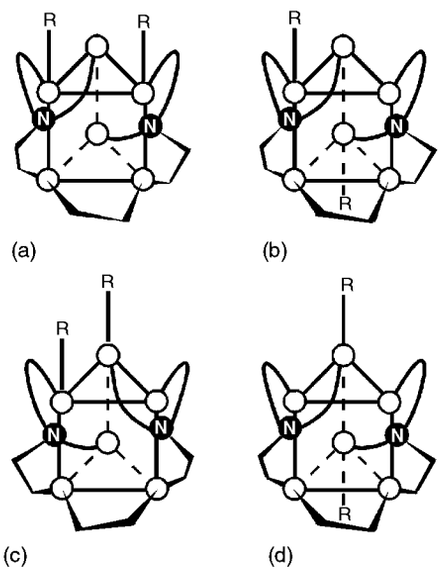

Fig. 5 Schematic representation of the possible isomers of the gadolinium complexes with the ligands **1**–**3**. For clarity reasons the water molecule capping the square plane is omitted.

and to the water binding site (Fig. 5). In the structure (d), featuring the two amide groups opposite to the water molecule, the geometry around the water site should be perturbed only very little with respect to the parent complex, whereas the position of the two substituents in the isomeric form (*a*) will have the largest influence. Thus, each isomeric form is endowed with a different τ_M and the observed value is likely to represent an average value. On this basis we may suggest that the shorter τ_M value for Gd·**2** be associated with a higher population of the structural form (d) in solution, favored by the high steric bulk of the isobutyl groups on the amide nitrogens.

NMRD profiles

The values of the other relaxation parameters can be calculated by the analysis of the magnetic field dependence of the proton relaxivity of the gadolinium(III) complexes (NMRD profiles; NMRD = Nuclear Magnetic Relaxation Dispersion) according to the equations for the inner- and outer-sphere relaxation mechanisms. The NMRD profiles were measured between 0.01 and 50 MHz at 6, 25 and 39 °C and at pH 7.2. The experimental profiles for Gd·**3** are shown in Fig. 6 together with the calculated curves with the best fitting parameters reported in Table 3. In the fitting procedure standard values for *a* (3.6 Å) and *D* (1.56×10^{-5}, 2.40×10^{-5} and 3.15×10^{-5} cm^2 s^{-1},[13] respectively) were used whereas τ_M at each temperature was fixed to the value calculated from the ^{17}O NMR data. The best fitting parameters show the expected temperature dependence and are in good agreement with those obtained for the parent complex and for other linear polyamino polycarboxylic complexes. The Gd–water proton distance for Gd·**2** is a little shorter than the value for the other two complexes but this difference could not be significant owing to its high covariance with τ_R and perhaps to the inadequacy in the evaluation of the outer sphere relaxivity. Thus, it appears that the only parameter that is significantly

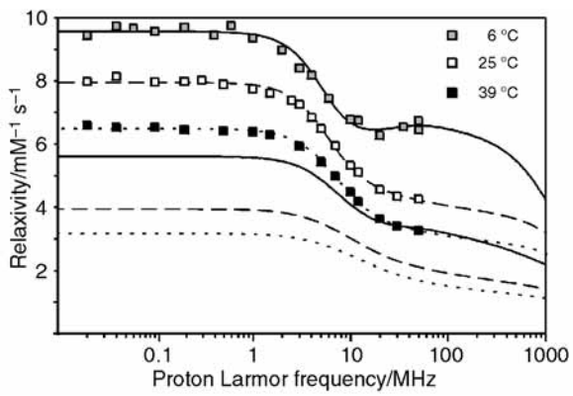

Fig. 6 $1/T_1$ NMRD profiles of an aqueous solution of Gd·**3** at pH 7.2 and at three different temperatures. The lower curves represent the calculated outer sphere contribution to proton relaxivity.

Table 4 Association constants and relaxivity of the adducts between Gd·**2**, Gd·**3** and bovine serum albumin at 25 °C and 20 MHz

Complex	K_A/M^{-1}	r_{1p}^b/mmol L^{-1} s^{-1}
Gd·**2**	1736	19.5
Gd·**3**	1398	43.2

influenced by the chemical substitution on egta is the exchange lifetime of the water molecule.

Formation of non-covalent adducts

The presence of hydrophobic groups on the amide nitrogen atoms of Gd·**2** and Gd·**3** enables the investigation of their non-covalent interaction with slowly tumbling substrates and establishment of the relaxivity enhancement attainable for the supramolecular adducts. In the presence of a binding interaction the reorientational correlation time of the complex substantially increases and the relaxivity is enhanced.[14–16] By titrating the complex with the substrate we can measure the successive increments in relaxivity that can be analysed according to the procedure of Proton Relaxation Enhancement (PRE) to give the association constant K_A and the relaxivity of the adduct, r_{1p}^b.[17] Solutions (0.02 mmol L^{-1}) of the complexes Gd·**2** and Gd·**3** were titrated with Bovine Serum Albumin and the increase of the relaxation rate measured at 20 MHz and 25 °C. The analysis of the data was done by assuming the presence of a single type of binding site on the protein and yielded the values listed in Table 4. The association constants are rather low compared to those measured for anionic complexes bearing benzylic groups,[16] but are not negligible. Gd·**2** shows a stronger interaction but a lower relaxation enhancement. This fact cannot be rationalized on the basis of the available data but it might be related to a different proportion of the structural isomers in solution for the two complexes and/or to a different mobility of the complex in the bound form. A value of 43.2 mmol L^{-1} s^{-1} of r_{1p}^b is similar to that observed for the analogous adducts with BSA of dota and dtpa derivatives and represents a limit imposed by the relatively long τ_M value.

Conclusion

The substitution of two carboxylates of egta with amide functionalities strongly influences the properties of the corresponding gadolinium(III) complexes. The ligands **1**–**3** present a much weaker chelating ability towards lanthanide cations and the thermodynamic stability of the complexes is reduced by about 3 orders of magnitude thus preventing any possible use *in vivo*. The complexes maintain one co-ordinated water molecule as in the parent compound but the increase of the net charge from −1 to +1 is accompanied by a decrease in the water exchange rate by an order of magnitude. On the other hand the k_{ex} values are similar to those of the anionic CAs $[Gd(dota)]^-$ and $[Gd(dtpa)]^{2-}$ and are about one order of magnitude higher than those of their corresponding neutral amide derivatives. The presence of hydrophobic substituents on the amide nitrogen atoms of Gd·**2** and Gd·**3** promotes their interaction with BSA to give adducts of enhanced relaxivity; however, the relatively long value of the exchange lifetime with respect to $[Gd(egta)]^-$ limits the relaxivity of the bound complexes and their positive charge results in a fairly weak affinity for the protein.

In order to functionalize the ligand with suitable substituents able to target macromolecular substrates, and yet maintaining the good chelating ability of egta and the optimal value of the water exchange rate of the gadolinium(III) complex, the substitution should occur either on the backbone of the ligand or on the acetic arms.

Experimental

Materials

The ligand H_4egta, Gd_2O_3, $GdCl_3·6H_2O$, isobutylamine and phenylpropylamine were purchased from Aldrich Chemical Co. The other reagents and solvent used were of commercially available reagent quality. Deoxygenation of solvents was carried out by bubbling N_2 through the solutions. Pyridine was distilled over NaOH under a N_2 atmosphere prior use. The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL EX400 FT spectrometer operating at 9.4 T.

Syntheses

egta bis(anhydride). 0.500 g (1.31 mmol) of H_4egta was suspended in 10 mL of freshly distilled and deoxygenated pyridine. After raising the temperature to 65 °C, 0.500 mL (5.25 mmol) of acetic anhydride was added over a period of 30 min and the mixture stirred for 20 h (Scheme 1). After cooling to room temperature the pyridine was removed under reduced pressure to give egta bis(anhydride) as a brownish oil (yield 98%). ^{1}H NMR ($CDCl_3$): δ 2.60 (t, *J* 5.7, NCH_2CH_2O, 4 H), 3.40 (s, OCH_2CH_2O, 4 H), 3.45 (t, *J* 5.7 Hz, NCH_2CH_2O, 4 H) and 3.66 (s, CH_2CO, 8 H). ^{13}C NMR ($CDCl_3$): δ 53.7 ($COCH_2N$), 54.9 (NCH_2CH_2O), 69.5 (NCH_2CH_2O), 70.3 (OCH_2CH_2O) and 165.9 (*C*O).

Ligand 1. To 15 mL of freshly distilled pyridine the egta bis(anhydride) (1.31 mmol) was added under vigorous stirring. NH_3 gas was bubbled over a period of 15 minutes through the solution maintained at 50 °C. After heating for 20 minutes, the solvent was evaporated under reduced pressure to give a yellowish oil which was redissolved in water. The pH of the aqueous solution was adjusted to 10 with NaOH and the solvent evaporated under reduced pressure. The resulting white solid (82%) was dried under vacuum at 50 °C. ^{1}H NMR (D_2O, pH 9.0): δ 2.88 (t, *J* 5.2 Hz, NCH_2, 4 H), 3.31 (s, H_2NCOCH_2, 4 H), 3.36 (s, $^-O_2CCH_2$, 4 H), 3.67 (t, *J* 5.2 Hz, CH_2O, 4 H) and 3.70 (s, OCH_2, 4 H). ^{13}C NMR (D_2O, pH 9.0): δ 55.78 (NCH_2CH_2O), 59.70 (H_2NCOCH_2N), 60.70 ($^-O_2CCH_2$), 70.10 (NCH_2CH_2O), 70.95 (OCH_2CH_2O), 179.49 and 180.74 (CO).

Ligand 2. To 10 mL of freshly distilled pyridine the egta bis(anhydride) (1.31 mmol) and 0.315 mL (3.13 mmol) of isobutylamine were added under vigorous stirring. The reaction mixture was stirred for 5 h at room temperature under a N_2 atmosphere. After evaporating the solvent under reduced pressure the residue was redissolved in water and the pH adjusted to 10 with NaOH. The resulting solution was extracted four times with diethyl ether (15 mL) while the pH was maintained at 10. The remaining aqueous solution was evaporated under reduced pressure and the white solid powder dried under vacuum at 50 °C (80%). ^{1}H NMR (D_2O, pH 9.5): δ 0.94 (d, 7.0 Hz, CH_3, 12 H), 1.84 (m, CH, 2 H), 2.87 (t, *J* 5.1 Hz, NCH_2, 4 H), 3.09 (d, *J* 6.9 Hz, CH_2NH, 4 H), 3.28 and 3.35 (s, $COCH_2N$, 4 H), 3.66 (t, *J* 5.1 Hz, CH_2O, 4 H) and 3.69 (s, OCH_2, 4 H). ^{13}C NMR (D_2O, pH 9.5) δ 20.8 (CH_3), 29.4 (CH), 47.9 (CH_2NH), 55.5 (NCH_2CH_2O), 60.0 ($HRNCOCH_2N$), 60.5 ($COCH_2N$), 70.7 (NCH_2CH_2O), 70.9 (OCH_2CH_2O), 175.8 and 180.6 (CO).

Ligand 3. To 10 mL of freshly distilled pyridine the egta bis(anhydride) (1.62 mmol) and 0.470 mL (3.24 mmol) of phenylpropylamine were added under vigorous stirring. The reaction mixture was stirred for 4 h at room temperature under a N_2 atmosphere. After evaporating the solvent under reduced pressure the residue was redissolved in water and the pH adjusted to 10 with NaOH. The resulting solution was extracted with diethyl ether (4 × 15 mL) while the pH was maintained at 10. The remaining aqueous solution was evaporated under reduced pressure and the white solid powder dried under vacuum at 50 °C (yield 78%). ^{1}H NMR (D_2O, pH 9.5): δ 1.79 (t, *J* 8.3 Hz, $PhCH_2$, 4 H), 1.90 (m, $PhCH_2CH_2$, 4 H), 2.62 (t, 8.2 Hz, CH_2CH_2NH, 4 H), 2.74 (t, *J* 7.7 Hz, NCH_2, 4 H), 3.22 and 3.24 (s, $COCH_2N$, 4 H), 3.47 (s, OCH_2, 4 H), 3.63 (t, *J* 7.7 Hz, CH_2O, 4 H) and 7.25 (m, C_6H_5, 10 H). ^{13}C NMR (D_2O, pH 9.5): δ 32.0, 34.7 and 41.5 (propylic moiety), 57.4 (NCH_2CH_2O), 58.2 ($HRNCOCH_2N$), 59.6 ($COCH_2N$), 66.8 (NCH_2CH_2O), 72.2 (OCH_2CH_2O), 128.5, 130.9, 131.0 and 144.3 (aromatic moiety), 175.9 and 180.7 (CO).

Gadolinium(III) complexes. The complexes were prepared by mixing stoichiometric amounts of the ligand and of gadolinium chloride and by adjusting the pH to 7 with NaOH. The solutions were kept at room temperature under vigorous stirring for about one hour until the pH stabilized. The compounds were then purified by precipitation by addition of acetone. The absence of free metal ion was assessed by the constant value of the water proton relaxation rate, measured at 298 K and 20 MHz, after successive additions of a small excess of the ligand.

^{1}H NMR Measurements

Water proton relaxation measurements (20 MHz, 25 °C) were carried out with a Stelar Spinmaster Spectrometer (Mede, Pv, Italy) on 0.5–2 mmol L^{-1} solutions of the complexes at pH 6.8, *I* 0.1 mol L^{-1}. Spin–lattice relaxation times T_1 were measured by the standard inversion recovery method with typical 90° pulse width of 3.5 ms, 16 experiments involving 4 scans. The reproducibility of the data is ±1%. The $1/T_1$ nuclear magnetic relaxation dispersion profiles of water protons were measured at 6, 25 and 39 °C using 1.5 mmol L^{-1} solutions of the complex on the field-cycling Koenig-Brown relaxometer of the University of Torino (Italy). The temperature was controlled by circulating a freon from an external bath and measured by a thermometer inserted into the freon close to the sample. The reproducibilities of the measured T_1 values were estimated to be ±2%.

The binding studies with BSA (fraction V, Merck) were carried out at pH 6.8 and 298 K on 0.2 mmol L^{-1} aqueous solutions of the gadolinium complexes prepared by dilution from 5 mmol L^{-1} stock solutions. Weighed amounts of BSA, corresponding to a concentration range of 0.15–1.8 mmol L^{-1}, were added to the solution under magnetic stirring. During the experiment, the pH of the solution did not change noticeably.

^{17}O NMR

Variable-temperature ^{17}O NMR measurements were recorded on a JEOL EX-90 (2.1 T) spectrometer, equipped with a 5 mm probe, by using a D_2O external lock. Experimental settings were: spectral width 10000 Hz, pulse width 7 μs, acquisition time 10 ms, 1000 scans and no sample spinning. Solutions containing 2.6% of ^{17}O isotope (Yeda, Israel) were used. The observed transverse relaxation rates (R°_{2obs}) were calculated from the signal width at half height.

Potentiometry and stability constant determination

The protonation and stability constants were determined by potentiometric titration at 25.0 ± 0.1 °C and $I = 0.1$ mol L^{-1} (KCl). The ligand solutions (25 mL, 0.001 mol L^{-1}) were titrated with a CO_2 free standard solution (0.1 mol L^{-1}) of KOH by using a Crison microburrette 2030 equipped with 2.500 mL syringe in a thermostatted cell under a stream of nitrogen. Potentiometric data were recorded by a Crison micropH 2002, equipped with a Metrohm combined glass elecrode and interfaced with a PC (NEWPASAT 2.00 software). The combined glass electrode was calibrated as a hydrogen concentration probe by titrating a known amount of HCl with 0.1 mol L^{-1} KOH and determining the equivalence point by Gran's method which allows the determination of the standard potential E° and of the ionic product of water K_W.[18] Complexation of Gd^{3+} with the present ligands is fast and the stability constants were determined by titration of the ligands in the presence of a known amount of $GdCl_3$ in ratios 1:1, 2:1 and 1:2. The cell temperature was controlled with a ISCO Crioterm GTR 190. At least three measurements (about 100 data points) were obtained for each system and fitted by the computer program SUPERQUAD 3.2.[19]

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