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Thermodynamic and structural properties of Gd^{3+} complexes with functionalized macrocyclic ligands based upon 1,4,7,10-tetraazacyclododecane

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The co-ordination properties toward Na^+, Ca^{2+}, Zn^{2+}, Cu^{2+} and Gd^{3+} of six ligands containing pendant functionalities attached to the common 1,4,7,10-tetraazacyclododecane macrocyclic ring have been studied by means of potentiometric and microcalorimetric methods. Stability constants for the 1,4,7,10-tetra(methylcarbamoylmethyl) ligand (DTMA), and for the new 10-(2-hydroxypropyl) 1,4,7-tri(2-hydroxymethylacetic acid) (HPDO3A-3HM) and 1,4,7-tri(2-hydroxymethylacetic acid) ligand (DO3A-3HM) are reported for the first time, while analogous data for the remaining ligands have been redetermined under the same experimental conditions (0.1 mol dm^{-3} NMe_4Cl, 298.1 ± 0.1 K). The synthesis of DTMA is also described. The stability of the GdL complexes follows the order DOTA (1,4,7,10-tetraacetic acid) ≈ HPDO3A [10-(2-hydroxypropyl) 1,4,7-triacetic acid] > DO3A (1,4,7-triacetic acid) > HPDO3A-3HM > DO3A-3HM > DTMA, although considering both complex formation and ligand basicity the binding selectivity of these ligands at physiological pH follows the order HPDO3A > DOTA > HPDO3A-3HM > DO3A-3HM > DO3A > DTMA. The thermodynamic resistance of the Gd^{3+} complexes, at a plasma concentration of clinical administrations, toward demetallation in the presence of important components of blood plasma, such as Na^+, Ca^{2+}, Cu^{2+}, Zn^{2+}, phosphate and carbonate, has been evaluated: the results are consistent with the previous trend of binding selectivity. The formation of intermediate complexed species formed in the first minutes of the complexation reactions has been studied from a thermodynamic point of view. The crystal structure of $[Gd(DTMA)(H_2O)][ClO_4]_3 \cdot NaClO_4 \cdot 3H_2O$, solved by X-ray analysis, displays the Gd^{3+} ion in a 9-co-ordinated environment defined by four nitrogens of the tetraazamacrocyclic moiety, eight amidic oxygen atoms of the side arms and by an oxygen atom of a water molecule.

Introduction

Over the last decade there has been an increasing interest in the chemistry of Gd^{3+} complexes because they have proved useful in diagnostic medicine as contrast agents for *in vivo* magnetic resonance imaging (MRI).[1–5] This technique furnishes images of anatomical districts which are topological representations of the ^1H NMR parameters, and hence the presence of the highly paramagnetic metal center, resulting in a large enhancement of water proton relaxation rate, improves the resolution of tissues.

Unfortunately, the applicability of Gd^{3+} complexes as contrast agents is strongly limited by their toxicity. The toxic effect of these metal complexes principally stems, apart from their intrinsic toxicity, from the metal ion and the ligand released before complex excretion. Numerous competitive equilibria, such as ligand protonation, Ca^{2+}, Zn^{2+}, Cu^{2+} complexation and precipitation of Gd^{3+} salts, to mention the principal ones, may contribute to the *in vivo* dissociation of similar Gd^{3+} complexes.

To avoid complex dissociation, Gd^{3+} chelates characterized by high thermodynamic stability and possibly kinetic inertness are commonly sought.[1] A convenient approach to obtain Gd^{3+} complexes with high thermodynamic stability has been the association of the high stability conferred to metal complexes by macrocyclic ligands with the neutralization of metal ion charge provided by negatively charged pendant groups.[5] Furthermore, macrocyclic ligands form metal complexes which are commonly very inert toward dissociation.

In addition the complex should present at least a water molecule in the first co-ordination sphere of the metal ion, in rapid exchange with the bulk solvent, in order efficiently to enhance the relaxation of solvent protons.[1]

Ligands such as DOTA, a twelve-membered tetraazamacrocycle bearing four carboxymethyl (acetate) pendant groups, and HPDO3A, the DOTA analogue containing only three acetate groups (Chart 1), combine these properties and have been successfully employed in the preparation of sufficiently safe contrast agents. Their Gd^{3+} complexes are presently in use in diagnostic MRI with the brand names DOTAREM™ and PROHANCE™, respectively.

The enhancement of water proton relaxation rate by Gd^{3+} complexes is ascribed to three principal phenomena, the first consisting in the exchange of co-ordinated water molecules with water molecules of the bulk solvent (*inner-sphere* contribution), the second involving the diffusion of water molecules in proximity of the paramagnetic center (*outer-sphere* contribution), and the last determined by prototropic exchange of mobile protons of the complex (*prototropic* contribution).[1,6] The contribution of prototropic exchange is commonly very difficult to assess, because this process is much slower than the exchange of co-ordinated water molecules.[7] The exchange lifetime of Gd^{3+}-co-ordinated water molecules, however, increases with decreasing negative charge of the Gd^{3+} complex, allowing the evaluation of the prototropic contribution.[6,8] For this reason Gd^{3+} complexes with neutral ligands have been considered. In a recent communication, dealing with the tripositive Gd^{3+} complex of DTMA (Chart 1), a distinct evaluation of water and prototropic exchange rates for a Gd^{3+}-co-ordinated solvent molecule was reported for the first time, evidencing that

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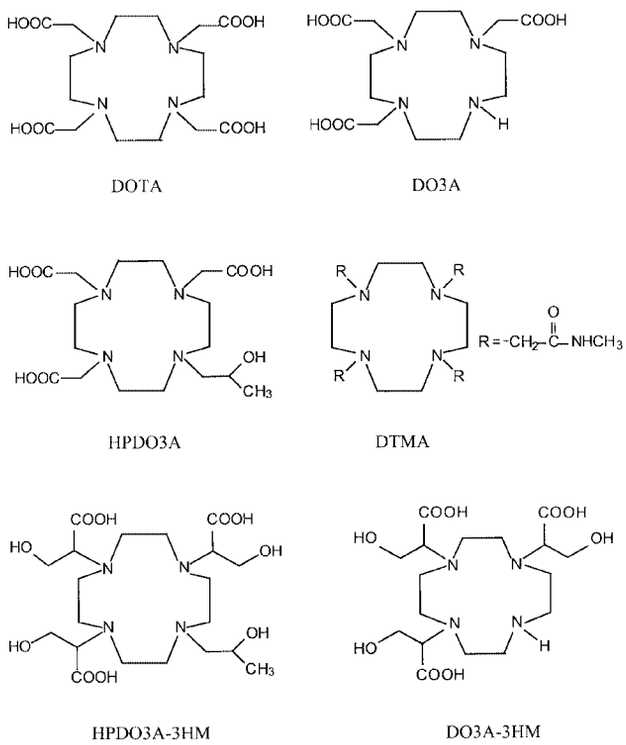

Chart 1

equivalent proton relaxivities can be achieved by exploiting different routes.[9]

Preliminary results concerning the speciation of the Gd^{3+}/DTMA system and the crystal structure of the $[Gd(DTMA)]^{3+}$ complex were reported in a successive communication from our laboratories, in which the pH dependence of relaxivity was interpreted in terms of the species formed in solution at equilibrium.[10] The use of the uncharged ligand, however, while allowing one to assess the contribution of prototropic exchange, leads to a Gd^{3+} complex of reduced stability.

In the present paper we report the results of a thermodynamic study performed to evidence how the loss of complex stability determined by the reduction of negative charge of ligands can be compensated by appropriate modifications of the ligand structures. To this purpose the six ligands reported in Chart 1 have been considered; two of them, HPDO3A-3HM and DO3A-3HM, were designed and synthesized intentionally.

The mechanism of Gd^{3+} complexation by similar ligands is commonly composed of different stages leading to the formation of intermediate species which undergo a slow reorganization to give the final complexes. In order to get a better understanding of the complexation process, we have studied the formation of such intermediate complexes with the considered ligands, by determining their stability constants and the relevant enthalpic and entropic parameters. This is the first microcalorimetric investigation on similar systems.

Finally, in the present paper we have also considered the competitive complexation equilibria with endogenous species, such as Na^+, Ca^{2+}, Zn^{2+} and Cu^{2+}, and precipitation of gadolinium(III) phosphate, carbonate and hydroxide to estimate the thermodynamic resistance of these metal complexes towards demetallation processes promoted by principal components of blood plasma.

Experimental

The compounds DOTA, DO3A and HPDO3A were synthesized as described.[5c,11] The synthesis of the new ligands HPDO3A-3HM and DO3A-3HM has been reported.[12] All these ligands were obtained in acidic form and characterized by means of elemental analysis, including determination of crystallization water by Karl Fischer's method, mass spectroscopy (ESI), ^1H and ^{13}C NMR, and HPLC, which verified the stoichiometry and purity. The lack of residual Na^+ in the samples was indicated by means of atomic absorption analysis.

Synthesis of DTMA

The synthesis of 1,4,7,10-tetrakis(methylcarbamoylmethyl)-1,4,7,10-tetraazacyclododecane (DTMA) was carried out by reaction of cyclen (1,4,7,10-tetraazacyclododecane) with 2-bromo-*N*-methylacetamide, according to a procedure adopted for similar ligands.[13]

2-Bromo-*N*-methylacetamide. (**CAUTION**: this compound is an irritant and must be handled with extreme care.) A solution of $BrCH_2COBr$ (34.7 cm^3, 400 mmol) in 1,2-dichloroethane (50 cm^3) was added over a period of 90 minutes, with vigorous stirring, to an aqueous solution of $MeNH_2$ (400 mmol, 34.7 cm^3) and NaOH (400 mmol, 16 g) at 10 °C. After the addition was complete, the suspension was maintained at this temperature for 50 minutes and then allowed to warm to room temperature. After filtration the organic phase was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to give a yellowish oil, which was dissolved in aqueous NaOH solution and extracted with diethyl ether. The ether solution was dried over anhydrous sodium sulfate, filtered and evaporated to dryness to give 24.5 g (40.3%) of a white solid. Calc. (found) for C_3H_6BrNO: C, 23.71 (23.5); H, 3.98 (4.0); N, 9.21 (9.0%). ^1H NMR ($CDCl_3$): δ 6.88 (s, NH, 1 H), 3.83 (s, CH_3, 3 H) and 2.80 (d, CH_2, 2 H). ^{13}C NMR ($CDCl_3$): δ 166.31 (s, CO, 1C), 28.94 (s, CH_3, 1C) and 26.83 (s, CH_2, 1C). MS: 58.1 *m*/*z* ($[O{\equiv}CNHMe]^+$) and 72.0 ($[CH_2{=}COHNHMe]^+$).

1,4,7,10-Tetrakis(methylcarbamoylmethyl)-1,4,7,10-tetraazacyclododecane (DTMA). 1,4,7,10-Tetraazacyclododecane (Fluka) (8.08 mmol, 1.4 g) was suspended in 200 cm^3 of anhydrous CH_3CN containing 80.8 mmol (14.2 cm^3) of diisopropylethylamine. To this suspension was added a solution of 2-bromo-*N*-methylacetamide (6.3 g, 41.45 mmol) in 150 cm^3 CH_3CN over a period of 5 h, at room temperature. The resulting solution was concentrated under reduced pressure and diethyl ether added to precipitate a first portion of pure DTMA, as a white solid, which was filtered off and dried in vacuum. The ether solution was evaporated to dryness and the crude product dissolved in the minimum quantity of chloroform and chromatographed (flash chromatography) on silica gel, eluting with a $CHCl_3$–MeOH–NH_3(30%) (6 : 3 : 0.5). The portions of DTMA were combined to give 2.7 g (66%) of pure compound. Calc. (found) for $C_5H_{10}N_2O$: C, 52.61 (52.5); H, 8.83 (8.8); N, 24.54 (24.4%). ^1H NMR ($CDCl_3$): δ 4.96 (s, NH, 1 H), 3.17 (s, CH_2CO, 8 H), 2.83 (s, CH_3, 12 H) and 2.77 (s, CH_2CH_2, 16 H). ^{13}C NMR ($CDCl_3$): δ 175.79 (s, CO, 4C), 60.53 (s, CH_2, 4C), 55.58 (s, CH_2CH_2, 8C) and 27.68 (s, CH_3, 4C). MS: *m*/*z* 457.57 ($[N + H]^+$).

Synthesis of $[Gd(DTMA)(H_2O)][ClO_4]_3{\cdot}NaClO_4{\cdot}3H_2O$

An aqueous solution (50 cm^3) of $[Gd(DTMA)]^{3+}$ was prepared by adding $GdCl_3$ (0.1 mmol) to a hot solution of DTMA (0.1 mmol). Crystals of $[Gd(DTMA)(H_2O)][ClO_4]_3{\cdot}NaClO_4{\cdot}3H_2O$ suitable for X-ray analysis were obtained by slow evaporation at room temperature of this solution after addition of $NaClO_4$ (0.5 g). Calc. (found) for $C_{20}H_{48}Cl_4GdN_8NaO_{24}$: C, 21.71 (21.6); H, 4.37 (4.3); N, 10.12 (10.1)%.

Crystal structure determination of $[Gd(DTMA)(H_2O)][ClO_4]_3{\cdot}NaClO_4{\cdot}3H_2O$

Crystal data. $C_{20}H_{48}Cl_4GdN_8NaO_{24}$, $M = 1106.70$, triclinic, $a = 11.558(6)$, $b = 13.251(9)$, $c = 14.713(9)$ Å, $\alpha = 94.29(9)$, $\beta = 109.65(8)$, $\gamma = 102.97(6)°$, $U = 2040(2)$ Å^3, $T = 298$ K, space

group $P\bar{1}$, (no. 2), $Z = 2$, μ(Mo-Kα) = 1.993 mm^{-1}. 7410 Reflections measured (up to $2\theta = 50$), 6377 with $I > 2\sigma(I)$, corrected for Lorentz-polarization effects, absorption correction by the Walker and Stuart method.[14] Direct methods (SIR 92[15]), full-matrix least squares (SHELXL 93[16]), atomic scattering factors and anomalous dispersion corrections for all atoms from ref. 17. Anisotropic displacement parameters for all the non-hydrogen atoms, hydrogen atoms of the macrocycle in calculated positions, with an overall thermal parameter factor refined up to 0.053(4) Å^2. The Fourier difference map did not allow us to localize the hydrogen atoms of the Gd co-ordinated and crystallization water molecules. Final $R[I > 2\sigma(I)]$ was 0.0496.

CCDC reference number 186/1797.

See http://www.rsc.org/suppdata/dt/a9/a909098c/ for crystallographic files in .cif format.

Potentiometric measurements

All pH-metric measurements (pH = $-\log$ [H$^+$]) employed for the determination of protonation and complexation constants were carried out in 0.1 mol dm^{-3} NMe_4Cl solutions at 298.1 ± 0.1 K, by using equipment and the methodology that has been described.[18] The combined Ingold 405 S7/120 electrode was calibrated as a hydrogen concentration probe by titrating known amounts of HCl with CO_2-free NaOH solutions and determining the equivalence point by Gran's method[19] which allows one to determine the standard potential $E°$ and the ionic product of water ($pK_w = 13.83(1)$ at 298.1 ± 0.1 K in 0.1 mol dm^{-3} NMe_4Cl). Empirical corrections were applied for the non-Nernstian response of the glass electrode below pH 2.5 and above pH 10.5.

At least three measurements (about 100 data points each one) were performed for each system in the pH ranges 2.0–12 for ligand protonation and 2.5–10.5 for complexation experiments. The ligand concentration [L] was about 1×10^{-3} mol dm^{-3} in all measurements while the metal ion concentration in the complexation experiments was 4[L] for Na^+ and 0.8[L]–2[L] for Ca^{2+}, Zn^{2+} and Cu^{2+}.

Complexation reactions of Gd^{3+} with the present ligands are slow; about four weeks are necessary to achieve the final equilibrium. For this reason out-of-cell experiments were performed in which 30 individual solutions (40 for DTMA), in the approximate pH range 2.5–6 (2.5–9 for DTMA), corresponding to single points of conventional titrations were stored in a thermostat at 298.1 ± 0.1 K, and their pH was periodically controlled to ensure the achievement of equilibrium conditions. For all ligands, DTMA excluded, the use of EDTA as an auxiliary ligand was necessary due to the very high value of the equilibrium constants to be determined. The variation of [H$^+$] with time clearly evidences a two step complexation reaction, in which an intermediate complex is formed in the first 25–50 minutes after mixing of ligand and metal ion, which develops towards the final, more stable complexes in a few weeks. The corresponding emf data were used to determine the equilibrium constants corresponding to both intermediate and final complex species. In the complexation experiments ligands (including EDTA) and metal ion concentrations were 1×10^{-3} mol dm^{-3}.

The computer program HYPERQUAD[20] was used to calculate the equilibrium constants from emf data.

Microcalorimetric measurements

The enthalpy changes for ligand protonation and metal ion complexation were determined in 0.1 mol dm^{-3} NMe_4Cl solutions by means of microcalorimetric titrations performed with an automated system composed of a Thermometric AB thermal activity monitor (model 2277) equipped with a perfusion-titration device and a Hamilton Pump (model Microlab M) coupled with a 0.250 cm^3 gas-tight Hamilton syringe (model 1750 LT). The measuring vessel was housed in a 25 dm^3 water thermostat which was maintained at the chosen temperature within $\pm 2 \times 10^{-4}$ K. The microcalorimeter was checked by determining the enthalpy of reaction of strong base (Me_4NOH) with strong acid (HCl) solutions. The value obtained, −13.55(5) kcal mol^{-1}, was in agreement with the literature values.[21] Further checks were performed by determining the enthalpies of protonation of ethylenediamine.

In a typical experiment, a Me_4NOH solution (0.1 mol dm^{-3}, addition volumes 15 μl) was added to acidic solutions (1.5 cm^3) of the ligands (5×10^{-3} mol dm^{-3}), containing Gd^{3+} (4×10^{-3} mol dm^{-3}) in the complexation experiments. Further complexation experiments were performed for each system by titrating ligand solution with Gd^{3+} solutions. Corrections for the heats of dilution were applied. The corresponding enthalpies of reaction were determined from the calorimetric data by means of a least squares fitting using the AAAL program.[22] No confident results were obtained for the fifth protonation step of DO3A and hence the experiments performed with this ligand were limited to the formation of the first four protonated species.

Speciation calculation for multi-equilibrium systems

The amounts of all species formed in the presence of Gd^{3+} complexes at a concentration of clinical administrations (2.5×10^{-5} mol dm^{-3}) and Na^+ (0.15 mol dm^{-3}), Ca^{2+} (2.5×10^{-5} mol dm^{-3}), Cu^{2+} (1.8×10^{-5} mol dm^{-3}), Zn^{2+} (1.5×10^{-5} mol dm^{-3}), phosphate (3.8×10^{-4} mol dm^{-3}) and carbonate (2.5×10^{-5} mol dm^{-3}) in blood plasma concentrations were calculated as a function of pH by means of the computer program HYSS,[23] including in the calculations the protonation constants of the ligands, the stability constants of the Gd^{3+}, Na^+, Ca^{2+}, Cu^{2+}, and Zn^{2+} complexes, the solubility products of gadolinium(III) phosphate, carbonate and hydroxide, and the protonation constants of phosphate and carbonate, all determined at 298.1 K.

Results and discussion

Description of the structure

Crystals of $[Gd(DTMA)(H_2O)][ClO_4]_3 \cdot NaClO_4 \cdot 3H_2O$ are made of $[Gd(DTMA)(H_2O)]^{3+}$ cations, sodium ions, perchlorate counter ions and crystallization water molecules. The macrocyclic ligand acts as octadentate. The metal ion is co-ordinated by the four nitrogens of the tetraazamacrocyclic moiety and by the amidic oxygen atoms provided by the side arms. The ninth position of the co-ordination sphere is occupied by an oxygen atom of a water molecule (Fig. 1). The Gd–N and the Gd–O amidic bond distances spanning in the ranges 2.621(5)–2.649(6) and 2.351(5)–2.455(5) Å, respectively, as well as the Gd–O (water) distance (2.461(5) Å) (Table 1) are comparable with those previously reported for analogous Gd^{3+} complexes.[25] The resulting polyhedron, which can be described as a distorted square antiprism,[26] capped by the water oxygen, corresponds to the usual co-ordination geometry of Gd^{3+} complexes with ligands based on the 1,4,7,10-tetraazacyclododecane structure.[25] Table 2 reports the dihedral angle values calculated for $[Gd(DTMA)(H_2O)]^{3+}$ together with those corresponding to an ideal (C_{4v}) monocapped square antiprism co-ordination environment. The four nitrogens and the four oxygen donor atoms, respectively, define two almost parallel mean planes (1.2(1)°). The oxygen atom of the Gd^{3+}-co-ordinated water molecule (O5) is *ca.* 1.7 Å out of the O1–O2–O3–O4 mean plane, on the opposite side of the metal ion, the Gd–O5 bond being almost perpendicular to this plane (87°). As already found,[5e,25b–d] the metal ion is closer to the oxygen donors' mean plane than to the plane described by the four nitrogens (0.74 *vs.* 1.59 Å).

The tetraaza ring has a [3333] C corners conformation,[27] with the four amidic chains in a head-to-tail relative arrangement (Fig. 1, lower). The four amidic nitrogen atoms have a significant sp^2 character, indicating that the usual π conjugation takes

Table 1 Primary co-ordination sphere bond distances (Å) and angles (°) for $[Gd(DTMA)(H_2O)]^{3+}$

Gd(1)–O(2)	2.351(5)	Gd(1)–N(4)	2.621(6)
Gd(1)–O(3)	2.351(4)	Gd(1)–N(1)	2.626(5)
Gd(1)–O(4)	2.393(5)	Gd(1)–N(3)	2.648(5)
Gd(1)–O(1)	2.455(5)	Gd(1)–N(2)	2.649(5)
Gd(1)–O(5)	2.461(6)		
O(2)–Gd(1)–O(3)	84.1(2)	O(5)–Gd(1)–N(1)	123.1(2)
O(2)–Gd(1)–O(4)	142.3(2)	N(4)–Gd(1)–N(1)	69.2(2)
O(3)–Gd(1)–O(4)	83.7(2)	O(2)–Gd(1)–N(3)	74.9(2)
O(2)–Gd(1)–O(1)	81.8(2)	O(3)–Gd(1)–N(3)	66.1(2)
O(3)–Gd(1)–O(1)	145.1(2)	O(4)–Gd(1)–N(3)	130.4(2)
O(4)–Gd(1)–O(1)	88.3(2)	O(1)–Gd(1)–N(3)	138.3(2)
O(2)–Gd(1)–O(5)	73.4(2)	O(5)–Gd(1)–N(3)	130.9(2)
O(3)–Gd(1)–O(5)	74.1(2)	N(4)–Gd(1)–N(3)	68.5(2)
O(4)–Gd(1)–O(5)	69.0(2)	N(1)–Gd(1)–N(3)	106.0(2)
O(1)–Gd(1)–O(5)	71.3(2)	O(2)–Gd(1)–N(2)	66.7(2)
O(2)–Gd(1)–N(4)	142.5(2)	O(3)–Gd(1)–N(2)	130.7(2)
O(3)–Gd(1)–N(4)	74.0(2)	O(4)–Gd(1)–N(2)	142.6(2)
O(4)–Gd(1)–N(4)	65.7(2)	O(1)–Gd(1)–N(2)	70.9(2)
O(1)–Gd(1)–N(4)	132.3(2)	O(5)–Gd(1)–N(2)	127.8(2)
O(5)–Gd(1)–N(4)	126.5(2)	N(4)–Gd(1)–N(2)	105.6(2)
O(2)–Gd(1)–N(1)	131.1(2)	N(1)–Gd(1)–N(2)	68.6(2)
O(3)–Gd(1)–N(1)	142.4(2)	N(3)–Gd(1)–N(2)	68.3(2)
O(4)–Gd(1)–N(1)	74.5(2)		
O(1)–Gd(1)–N(1)	65.4(2)		

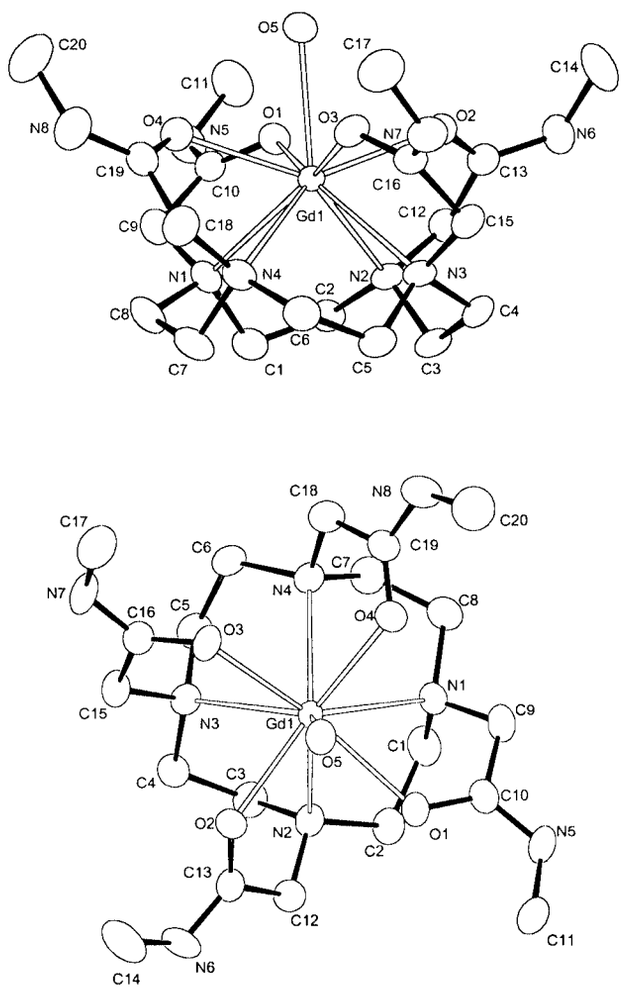

Fig. 1 ORTEP[24] drawings of the $[Gd(DTMA)(H_2O)]^{3+}$ cation.

place in each amidic functional group thus enhancing the donor capability of the carbonylic oxygens.

Concerning the packing, it is worth noting that the oxygen of the Gd^{3+}-co-ordinated water molecule is very close (2.92(1) Å) to an oxygen atom of a perchlorate ion, suggesting that a strong hydrogen bond between $[Gd(DTMA)(H_2O)]^{3+}$ and a counter ion occurs. In addition the water oxygen O5 is 3.483(7) Å from a symmetry related O5 atom.

Finally a lot of hydrogen-bond contacts (<3.0 Å) involving the crystallization water molecules, the oxygen atoms of the perchlorate anions and various hydrogen and nitrogen atoms of the ligand are present.

The coprecipitated Na^+ ion is six-co-ordinated by four perchlorate oxygens and two crystallization water molecules, the Na–O bond lengths ranging from 2.413(9) to 2.80(1) Å.

Table 2 Dihedral angles (°) for $[Gd(DTMA)(H_2O)]^{3+}$

Face 1	Face 2	Angle/°	Idealized angle for a C_{4v} geometry
N3–O2–O3	N1–O1–O4	155.2(2)	163.5
N4–O3–O4	N2–O1–O2	157.6(2)	163.5
N3–N4–O3	N1–N2–O1	142.1(2)	138.2
N2–N3–O2	N1–N4–O4	145.1	138.2
N1–N3–N4	N1–N2–N3	0.3(2)	0.0

Ligand protonation studies in solution

The protonation constants of the six ligands considered in this study, determined in 0.1 mol dm^{-3} NMe_4Cl solutions at 298.1 ± 0.1 K, are listed in Table 3. The log K values obtained for DOTA, DO3A and HPDO3A follow the same trend reported by other authors,[5d,28,29] the first two protonation steps taking place above neutral pH and the remaining ones below neutral pH. In the case of DOTA our log K values are in good agreement with previously reported data[28] obtained under the same experimental conditions, although a small difference in the first protonation constant is observed. In the case of DO3A all our protonation constants, but the first one, well compare with previous values.[29] The literature value of the first protonation constant (log $K = 11.59$) is considerably lower than that we found (12.46(1)). Several measurements in very alkaline solutions (up to pH 12) were performed to verify our value after accurate calibration of the glass electrode in such media (see Experimental section). Significant differences were also found for the protonation constants of HPDO3A with respect to the literature values (log $K_i = 11.96$, 9.43, 4.30, 3.26).[29] Also in this case, considerable experimental work was performed to ensure the purity of the ligand employed and the accuracy of the potentiometric measurements (see Experimental section).

The compound DTMA displays a largely reduced ability to bind H^+ ions with respect to the other ligands, evidencing the effect of the negatively charged groups on the basicity of such ligands. Actually, carboxylate groups, in addition to their intrinsic basicity, can assist protonation of ligand amine groups *via* hydrogen bonding.

Finally HPDO3A-3HM and DO3A-3HM are characterized, in each protonation step, by a moderately lower basicity with respect to the other negatively charged ligands, which can be ascribed to some impediment in the stabilization of the protonated species *via* intramolecular hydrogen bonding, determined by the increased ligand rigidity produced by the bulky pendant arms.

Interesting information about the ligand protonation reactions is furnished by the enthalpy and entropy changes determined for these proton transfer processes. As shown in Table 3, protonations of the considered ligands are promoted by both favorable enthalpic ($\Delta H° < 0$) and entropic ($T\Delta S° > 0$) contributions, with the unique exceptions of the entropic changes for the last protonation step of DTMA and DO3A-3HM. Concerning the enthalpic contributions, it is well known that amine protonation is a markedly exothermic reaction, while protonation of acetate groups is an almost athermic reaction.[21] Accordingly, considering the enthalpy changes listed in Table 3,

Table 3 Thermodynamic parameters for ligand protonation reactions, determined in 0.1 mol dm^{-3} NMe_4Cl solutions at 298.1 ±0.1 K. $\Delta H°$ and $T\Delta S°$ in kcal mol^{-1}

	DOTA			DO3A			HPDO3A		
Reaction	log K	$-\Delta H°$	$T\Delta S°$	log K	$-\Delta H°$	$T\Delta S°$	log K	$-\Delta H°$	$T\Delta S°$
$H + L \rightleftharpoons HL$ [a]	11.74(4) [b]	11.9(2)	4.1(2)	12.46(1)	8.1(1)	8.5(1)	11.17(1)	11.8(1)	3.4(1)
$H + HL \rightleftharpoons H_2L$	9.76(4)	9.5(2)	3.8(2)	9.49(1)	10.0(2)	2.9(2)	9.33(1)	9.8(1)	2.9(1)
$H + H_2L \rightleftharpoons H_3L$	4.68(4)	2.5(2)	3.9(2)	4.26(1)	1.8(2)	3.9(2)	4.99(1)	4.9(2)	1.9(2)
$H + H_3L \rightleftharpoons H_4L$	4.11(4)	3.0(2)	2.6(2)	3.51(1)	2.7(2)	2.1(2)	3.80(1)	4.1(2)	1.1(2)
$H + H_4L \rightleftharpoons H_5L$	2.37(5)	1.7(2)	1.5(2)	1.97(1)	[c]	[c]	2.84(1)	1.6(2)	2.3(2)
	DTMA			HPDO3A-3HM			DO3A-3HM		
Reaction	log K	$-\Delta H°$	$T\Delta S°$	log K	$-\Delta H°$	$T\Delta S°$	log K	$-\Delta H°$	$T\Delta S°$
$H + L \rightleftharpoons HL$	9.27(1)	11.04(4)	1.60(4)	10.68(2)	5.1(1)	9.5(1)	10.93(1)	9.5(2)	5.4(2)
$H + HL \rightleftharpoons H_2L$	5.55(2)	6.78(4)	0.79(4)	7.81(3)	5.8(1)	4.8(1)	7.08(2)	5.1(2)	4.5(2)
$H + H_2L \rightleftharpoons H_3L$	1.56(7)	4.0(3)	−1.9(3)	4.14(4)	3.2(1)	2.4(1)	4.04(2)	2.2(3)	3.3(3)
$H + H_3L \rightleftharpoons H_4L$				3.37(5)	1.9(1)	2.7(1)	3.49(2)	5.1(3)	−0.3(3)

[a] Charges omitted. [b] Values in parentheses are standard deviations in the last significant figure. [c] Not determined.

it is clear that the first two protonations occur at the amine groups of the ligands, although the acetate residues are capable of contributing to the stability of such protonated species by hydrogen bonding and charge–charge interaction. In the case of DTMA also the third protonation takes place necessarily on an amine group in accordance with the exothermic character of the reaction. Conversely, for the other ligands most of the following protonation reactions are considerably less exothermic indicating a principal involvement of carboxylate groups in the last protonation steps. Such a protonation sequence (the first two protons binding nitrogen atoms and the successive ones binding carboxylate groups) was clearly evidenced for DOTA by means of NMR studies in solution.[30] Accordingly, the crystal structure of $DO3A \cdot H_2SO_4$ displayed only two out of the five acidic protons bound to nitrogen atoms, the remaining three H^+ ions being linked to carboxylate groups.[29] The structure also revealed that two *trans*annular nitrogen atoms are involved in ligand protonation one of which is the secondary one.

Only in the case of HPDO3A the third and the fourth protonations are rather exothermic suggesting that for this ligand a significant participation of amine groups in the binding of H^+ ions occurs up to the fourth protonation step. This behavior is rather surprising for a polyaminopolycarboxylic ligand based upon 1,4,7,10-tetraazacyclododecane, resembling the features of analogous ligands containing larger tetraazamacrocyclic moieties.[30b] Nevertheless, also the fourth protonation step of DO3A-3HM is significantly more exothermic than expected on the basis of the general trend of decreasing enthalpic contributions with increasing number of protons on the ligands, in agreement with a third proton binding to the tetraazamacrocyclic moiety at the fourth protonation stage.

The favorable entropic contributions to the protonation of these ligands are mainly determined by desolvation processes due to the charge neutralization occurring upon proton binding by the ligand. On the other hand the increasing stiffening of these cyclic molecules, occurring upon successive ligand protonation, determined by the presence of charged ammonium groups in close proximity to each other and by the formation of intramolecular hydrogen bonds involving the ammonium (amine) groups and the carboxylate (carboxylic) residues, results in a loss of entropy which is reflected by the lower entropic contributions observed in the last protonation steps. In this sense, DTMA represents a particular case since it does not contain charged functionalities. In such a case desolvation processes are less important, and the entropic contributions to DTMA protonation are very small, becoming unfavorable ($T\Delta S° < 0$) for the last protonation step.

Gd^{3+} Complexation in solution

The stability constants of the Gd^{3+} complexes with DTMA, HPDO3A-3HM and DO3A-3HM are reported in Table 4 together with those for DOTA, DO3A and HPDO3A we have redetermined by means of our method, under our experimental conditions. Very good agreement is found with a literature value (log $K = 24.6$)[5a] of the stability constant of $[Gd(DOTA)]^-$ (other values obtained at 25 °C and 0.1 mol dm^{-3} ionic strength are log $K = 24.0$,[5d] 25.3[30a]) while moderately higher values for the complexes Gd(DO3A) and Gd(HPDO3A) (log K = 22.02(2), 24.5(1); Table 4) were found with respect to the literature values (log $K = 21.0$, 23.8).[28]

As can be seen from Table 4, the stability of the Gd^{3+} complexes is strongly affected by the nature of the pendant functionalities linked to the common tetraazamacrocyclic ring.

The complex of lower stability is formed by DTMA, the ligand bearing four uncharged amidic groups. According to the structure of $[Gd(DTMA)(H_2O)]^{3+}$ observed in the solid state (Fig. 1), it seems likely that also in solution the metal ion is coordinated by the four nitrogen atoms of the macrocyclic moiety and by the four amidic oxygen atoms provided by the side arms, the usual nine-co-ordination being completed by a solvent water molecule. Starting from neutral pH this metal bound water molecule dissociates ($pK_a = 7.9(1)$) giving rise to the hydroxo complex $[Gd(DTMA)(OH)]^{2+}$, while in acidic solutions (pH < 5) $[Gd(DTMA)]^{3+}$ undergoes protonation producing the species $[Gd(HDTMA)]^{4+}$. Owing to the modest co-ordinating ability of DTMA toward Gd^{3+}, unco-ordinated metal ion is present in acidic media up to pH 4.

Considerably more stable complexes are formed by the ligands bearing charged pendant functionalities. Among these, the fully deprotonated forms of DOTA and HPDO3A form the most stable complexes, the stability constants of $[Gd(DOTA)]^-$ and Gd(HPDO3A) being equal within the experimental errors, in spite of the lower negative charge of HPDO3A. On the other hand, Gd(DO3A) displays a significantly lower stability than that of Gd(HPDO3A) (Table 4), although both ligands (DO3A and HPDO3A) have the same negative charge in the complexes. Evidently, the presence of three negative charges instead of four on the ligand does not influence the stability of the Gd^{3+} complex, while the loss of a donor atom has a considerable effect in lowering the complex stability. Actually, both DOTA and HPDO3A bind Gd^{3+} producing a N_4O_4 co-ordination environment, similar to that displayed by DTMA (Fig. 1), while a N_4O_3 set of donors is furnished by DO3A, as demonstrated in the solid state by crystal structures of the complexes.[25c,d,28]

Table 4 Stability constants of Gd^{3+} complexes determined in 0.1 mol dm^{-3} NMe_4Cl solutions at 298.1 ± 0.1 K

Reaction	log *K*					
	DOTA	DO3A	HPDO3A	DTMA	HPDO3A-3HM	DO3A-3HM
$Gd + L \rightleftharpoons GdL$ [a]	24.67(7) [b]	22.02(2)	24.5(1)	12.8(1)	19.4(1)	18.82(9)
$GdL + H \rightleftharpoons Gd(HL)$				3.4(1)		
$GdL + OH \rightleftharpoons GdL(OH)$				5.8(1)		2.63(1)

[a] Charges omitted. [b] Values in parentheses are standard deviations in the last significant figure.

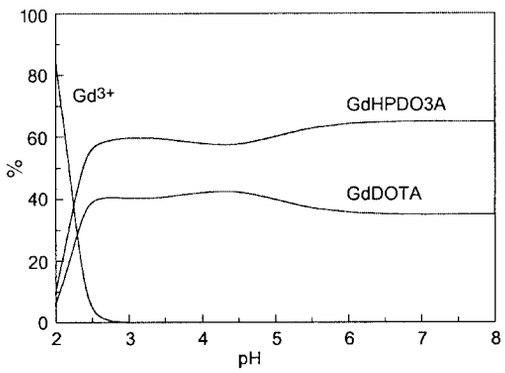

Fig. 2 Percentages of Gd^{3+} bound to DOTA and to HPDO3A calculated as a function of pH for equimolar concentrations (1×10^{-3} mol dm^{-3}) of reactants.

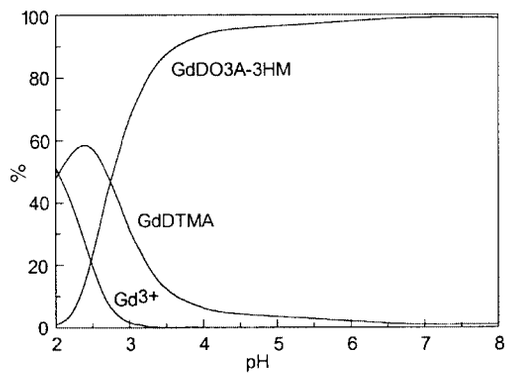

Fig. 3 Percentages of Gd^{3+} bound to DTMA and to DO3A-3HM calculated as a function of pH for equimolar concentrations (1×10^{-3} mol dm^{-3}) of reactants.

The compound HPDO3A-3HM is ideally obtained by inserting three CH_2OH groups on the side arms of HPDO3A bearing the carboxylate residues. Hence, with respect to HPDO3A, HPDO3A-3HM contains three additional oxygen atoms which might act as donor atoms toward Gd^{3+}, enhancing the complex stability. Conversely, the stability of the Gd(HPDO3A-3HM) (log $K = 19.4$) complex is considerably lower that that displayed by Gd(HPDO3A) (log $K = 24.5$). This is due, most likely, to a greater steric hindrance of the hydroxypropanoic functionalities with respect to the acetic ones, which determines a more strained, less stable, co-ordination environment of the metal ion. On the other hand, HPDO3A-3HM contains a surplus of donor atoms and elimination of the hydroxypropyl group, to give DO3A-3HM, does not result in a significant loss of complex stability (log $K = 18.82$ for Gd(DO3A-3HM)), as otherwise observed upon eliminating the hydroxypropyl group of HPDO3A to give DO3A.

As far as the selectivity of the present ligands in the binding of Gd^{3+} in aqueous solution is considered, it is interesting that the simple analysis of the complex stability constants does not lead to correct interpretations. Recently, it has been shown that a useful method to determine thermodynamic selectivity trends in the co-ordination properties of couples of ligands consists in the calculation of the percentages of metal bound to each ligand, as a function of pH, for solutions containing the ligands and the metal ion in equimolar amounts.[31] A similar calculation, performed for the systems Gd^{3+}/DOTA/HPDO3A (all components 1×10^{-3} mol dm^{-3}) by using the complex stability constants and the ligand protonation constants reported in Tables 4 and 3, respectively, furnishes the diagram in Fig. 2, evidencing a greater propensity of HPDO3A to bind Gd^{3+}. By performing similar calculations for all possible couples of ligands we obtain the trend of binding selectivity HPDO3A > DOTA > HPDO3A-3HM > DO3A-3HM > DO3A > DTMA over all the pH range considered (2–8). Only for the Gd^{3+}/DTMA/DO3A-3HM system (Fig. 3) inversion of selectivity between the two ligands is observed in acidic solution: while for pH > 3 Gd^{3+} is preferentially bound to DO3A-3HM, at lower pH the DTMA complexes prevail due to the formation of the monoprotonated $[Gd(HDTMA)]^{4+}$ species.

It is interesting that in spite of the higher stability constants of the Gd^{3+} complex displayed by DO3A, HPDO3A-3HM and DO3A-3HM demonstrate a greater ability in the binding of the metal ion in the pH range considered (2–8). This is due to the higher basicity of DO3A, which determines a stronger competition between metal ion complexation and ligand protonation. On the other hand, predominant binding of Gd^{3+} by DO3A is expected in very alkaline solutions where ligand protonation does not compete with Gd^{3+} complexation.

Demetallation of Gd^{3+} complexes by endogenous agents

As mentioned in the introduction, the toxicity of Gd^{3+} complexes is principally determined by the release of the highly toxic Gd^{3+} ion. In order to forecast the fate of such compounds in the case of *in vivo* administration, some computer models of blood plasma have been developed in an attempt to simulate all the equilibria that are expected to take place in this medium. To define a good model of blood plasma for a gadolinium complex, all species contained in blood plasma which are known to bind Gd^{3+}, to form different complexes with the examined ligand, and produce insoluble compounds with the components of the complex, or the complex itself, should be considered. Different models considering as many equilibria as possible [32,33] or adopting simplified sets of equilibria [34] have been proposed. Nevertheless, the potential applicability of such simulation efforts is presently limited by two main factors: (*i*) the absence of complete sets of equilibrium data consistent with the physiological conditions; (*ii*) the influence of the physiological regulatory systems that oppose, *in vivo*, the equilibrium shifts predicted by calculation based upon a static model. In addition such thermodynamic models do not consider the kinetic aspects connected with the demetallation and/or transmetallation reactions. From this point of view Gd^{3+} complexes with functionalized macrocyclic ligands are favorably sluggish, and predictions based on such thermodynamic models are generally pessimistic considering the time required for complex excretion.

In the present work, in order to evaluate the amounts of Gd^{3+} released, at equilibrium, by the considered complexes in solutions containing important components of blood plasma,

Table 5 Stability constants of Na^+, Ca^{2+}, Zn^{2+} and Cu^{2+} complexes determined in 0.1 mol dm^{-3} NMe_4Cl solutions at 298.1 ± 0.1 K

	log *K*					
Reaction	DOTA	DO3A	HPDO3A	DTMA	HPDO3A-3HM	DO3A-3HM
$Na + L \rightleftharpoons NaL$ [a]	4.03(4) [b]	2.20(2)	3.19(3)		2.50(1)	2.58(3)
$Ca + L \rightleftharpoons CaL$	17.219(8)	13.39(2)	14.06(1)	9.47(3)	11.09(4)	8.84(2)
$CaL + H \rightleftharpoons Ca(HL)$	3.80(4)		5.38(2)	3.9(1)		5.80(7)
$Ca(HL) + H \rightleftharpoons Ca(H_2L)$	3.77(9)					5.54(7)
$CaL + 2H \rightleftharpoons Ca(H_2L)$	7.57(9)	11.36(5)				11.34(7)
$Ca(H_2L) + H \rightleftharpoons Ca(H_3L)$		3.8(1)				
$CaL + OH \rightleftharpoons CaL(OH)$				2.9(8)		5.94(7)
$CaL + Ca \rightleftharpoons Ca_2L$					3.70(5)	
$Zn + L \rightleftharpoons ZnL$	20.52(1)	21.87(3)	20.41(8)	13.00(3)	17.16(2)	17.20(1)
$ZnL + H \rightleftharpoons Zn(HL)$	4.35(1)	3.27(2)	4.92(7)		4.23(2)	3.51(1)
$Zn(HL) + H \rightleftharpoons Zn(H_2L)$	3.49(1)	2.81(6)	3.13(8)			
$ZnL + 2H \rightleftharpoons Zn(H_2L)$	7.84(1)	6.08(6)	8.05(8)			
$ZnL + OH \rightleftharpoons ZnL(OH)$		2.39(7)		3.75(5)	3.1(1)	8.44(6)
$ZnL(OH) + OH \rightleftharpoons ZnL(OH)_2$				3.71(4)		
$Cu + L \rightleftharpoons CuL$	22.44(9)	21.65(2)	24.19(3)	14.12(3)	19.32(3)	19.31(5)
$CuL + H \rightleftharpoons Cu(HL)$	4.31(9)	3.86(2)	4.61(4)		4.11(3)	3.49(5)
$Cu(HL) + H \rightleftharpoons Cu(H_2L)$	3.52(9)					2.23(8)
$CuL + 2H \rightleftharpoons Cu(H_2L)$	7.83(9)					
$CuL + OH \rightleftharpoons CuL(OH)$				5.46(4)		8.18(8)
$CuL(OH) + OH \rightleftharpoons CuL(OH)_2$				3.32(5)		

[a] Charges omitted. [b] Values in parentheses are standard deviations in the last significant figure.

such as Na^+, Ca^{2+}, Cu^{2+}, Zn^{2+}, phosphate and carbonate, the concentrations of all species formed in the presence of Gd^{3+} complexes at a plasma concentration of clinical administrations (2.5×10^{-5} mol dm^{-3}) were calculated as a function of pH by means of the computer program HYSS.[23] To perform such calculations, we have determined the stability constants of the Na^+, Ca^{2+}, Cu^{2+}, and Zn^{2+} complexes with DTMA, HPDO3A-3HM and DO3A-3HM, and redetermined under our experimental conditions the stability constants of the complexes formed by DOTA, DO3A, and HPDO3A with the same metal ions (Table 5).

According to the calculations, the percentages of Gd^{3+} complex which are retained at physiological pH 7.4 are 93.9% for HPDO3A, 93.8% for DOTA, 92.4% for HPDO3A-3HM, 89.1% for DO3A-3HM, 87.8% for DO3A and 84.8% for DTMA. The released Gd^{3+} precipitates in the form of $GdPO_4$, while gadolinium carbonate and hydroxide are not formed. On the other hand the released ligand is almost totally involved in the co-ordination to Cu^{2+}, Zn^{2+} and Ca^{2+}.

It is to be stressed here that such calculations do not pretend to produce a biospeciation in presence of similar Gd^{3+} complexes but, in spite of the use of equilibrium data obtained at 298.1 K and the ignored equilibria involving further endogenous agents, such as amino acids, citrate and human serum albumin, they furnish a reasonable estimation of the thermodynamic resistance of these metal complexes towards demetallation processes promoted by principal components of blood plasma. In addition such calculations can be performed by means of a readily available computer program (HYSS).[23]

It is interesting that the calculated percentages of undissociated Gd^{3+} complexes are consistent with the trend of selectivity, displayed by these ligands in the binding of Gd^{3+} at physiological pH, discussed in the previous section. This means that the thermodynamic properties of these complex systems (complex stability and ligand basicity) are of major importance in determining the applicability of such complexes as contrast agents.

Formation of intermediate species in complexation reactions

It has been shown by kinetic studies that complexation of Gd^{3+} by polyaminopolycarboxylic macrocyclic ligands proceeds through the formation of intermediate complexes, in which the ligands should be in monoprotonated form and the metal ion is expected to be co-ordinated by the carboxylate groups and at least one nitrogen atom of the ligand, followed by its slow reorganization.[5f,35]

In a recent communication[36] some of us reported that by means of potentiometric (pH-metric) measurements it is possible to evidence different stages in these complexation reactions of Gd^{3+} with DOTA and DO3A. Now we have extended this study to HPDO3A, DTMA, HPDO3A-3HM and DO3A-3HM. The additions of Gd^{3+} to solutions containing protonated forms of these ligands produces a rapid jump of H^+ concentration due to the release of protons by the ligand during metal ion complexation. After this almost instantaneous process the solution pH evolves in about 25–50 minutes towards a seemingly constant value, although three to four weeks are necessary to attain the final equilibrium corresponding to the complex stability constants in Table 4. Potentiometric data obtained for the 25–50 minutes equilibration evidenced that, at this stage, both monoprotonated and unprotonated intermediate complexes are present in solution for all ligands, but for HPDO3A and DO3A-3HM which do not form monoprotonated complexes, while also diprotonated species are formed with HPDO3A-3HM and DO3A-3HM, allowing the determination of the relevant stability constants listed in Table 6. In order to get insight into the nature of the metal to ligand interaction at this intermediate stage, we have also measured the enthalpy changes associated to the formation of these complexes (Table 6).

As far as the unprotonated complexes are considered, the results obtained indicate that, with the exception of DTMA, these intermediate species are very stable, the difference in stability between the intermediate and the final ones being rather small. In energetic terms, 80–90% of the free energy gain ($\Delta G^\circ = -RT \log K$) associated with the attainment of the final complexation stages (93% in the case of HPDO3A) is produced in the first minutes of these long processes lasting over three weeks. A similar behavior suggests that a good co-operativity between carboxylate and amine groups in metal ion chelation occurs also in the formation of the intermediate complexes. Nevertheless, important differences in the complexation modes are revealed by the enthalpic contributions to complexation. Actually the formation of such Gd^{3+} intermediate complexes is promoted by favorable enthalpic contributions

Table 6 Thermodynamic parameters for intermediate equilibria of Gd^{3+} complexation determined in 0.1 mol dm^{-3} NMe_4Cl solutions at 298.1 ± 0.1 K

	DOTA			DO3A			HPDO3A		
Reaction	log K*	$-\Delta H°$*	$T\Delta S°$*	log K*	$-\Delta H°$*	$T\Delta S°$*	log K*	$-\Delta H°$*	$T\Delta S°$*
$Gd + L \rightleftharpoons GdL$ [a]	21.2(1) [b]	6.8(2)	22(1)	19.6(1)	4.64(8)	22.1(2)	22.9(1)	11.2(3)	20.0(3)
$Gd + L + H \rightleftharpoons Gd(HL)$	24.5(1)	10.2(1)	23.2(4)	22.5(1)	11.24(8)	19.5(3)			
$GdL + H \rightleftharpoons Gd(HL)$	3.3(1)	3.4(3)	1.0(4)	2.9(2)	6.6(1)	−2.6(4)			
	DTMA			HPDO3A-3HM			DO3A-3HM		
$Gd + L \rightleftharpoons GdL$	8.6(1)	10.1(2)	1.6(2)	15.2(1)	−3.0(1)	23.7(1)	15.5(1)	−0.1(1)	21.2(1)
$Gd + L + H \rightleftharpoons Gd(HL)$	13.2(1)	2.9(3)	15.1(3)	19.8(1)	3.1(1)	23.9(1)			
$Gd + L + 2H \rightleftharpoons Gd(H_2L)$				22.8(1)	8.6(2)	22.5(2)	22.1(1)	10.2(1)	19.9(1)
$GdL + H \rightleftharpoons Gd(HL)$	4.6(1)	−7.2(3)	13.5(3)	4.6(1)	6.1(1)	0.2(1)			
$Gd(HL) + H \rightleftharpoons Gd(H_2L)$				2.9(1)	5.5(2)	−1.4(2)			
$GdL + 2H \rightleftharpoons Gd(H_2L)$				7.6(1)	11.6(2)	−1.2(2)	6.6(1)	10.3(1)	−1.3(1)

[a] Charges omitted. [b] Values in parentheses are standard deviations in the last significant figure.

($\Delta H° < 0$) in the case of DOTA, DO3A, HPDO3A and DTMA, an unfavorable contribution ($\Delta H° > 0$) is found for HPDO3A-3HM and no enthalpic contribution is observed with DO3A-3HM.

Similarly to the case of ligand protonation, also metal ion complexation involves the formation of binding interactions (co-ordinative bonds) and desolvation of the reacting species. From an enthalpic point of view these processes furnish opposite contributions, the first one being favorable and second unfavorable. The extent of desolvation primarily depends on the neutralization of charge occurring upon interaction of the metal ion with the donor atoms, and hence the negatively charged acetate groups result in a larger release of solvent molecules than the uncharged amine groups. On the other hand, a large desolvation corresponds to a very favorable entropic contribution. This is the reason why metal ion co-ordination by carboxylates is promoted by the entropic term, the enthalpic one being unfavorable or almost negligible, while amine groups give rise to complexation reactions with favorable enthalpy changes.[21]

Based on these considerations, the thermodynamic parameters reported in Table 4 are strongly indicative of the fact that in the intermediate Gd^{3+} complexes with HPDO3A-3HM and DO3A-3HM the ligand carboxylate groups give the principal contribution to complex stability, while for the remaining ligands co-ordination of amine nitrogens is more important. As already said, the intermediate complexes of HPDO3A-3HM and DO3A-3HM can bind up to two protons. The enthalpy changes obtained for these protonation processes (Table 6) are typical of unco-ordinated amine groups, indicating that at least two nitrogen atoms of the macrocyclic backbone are not involved in the co-ordination. Most likely, at this preliminary complexation stage, HPDO3A-3HM and DO3A-3HM hold the metal ion in the region of the pendant functionalities, thanks to the presence of a large number of donor atoms, and probably only one amine group takes part in complexation. Considering the protonation properties of the twelve-membered tetraazamacrocyclic moiety it seems improbable that a greater number of amine groups can participate.

Conversely, the intermediate Gd^{3+} complexes with the remaining ligands are expected to have a structure, more similar to that of the final species. These complexes display a considerably lower tendency to bind protons in solution with respect to the analogous species formed by HPDO3A-3HM and DO3A-3HM (Table 6). The compound HPDO3A does not undergo protonation in the pH range considered (pH > 2.5), while for DOTA and DO3A only monoprotonated complexes are formed in very acidic solutions, and the favorable enthalpy changes ($\Delta H° < 0$) for such protonation reactions indicate that also in this case protonation occurs at free amine nitrogens. Hence no more than one amine nitrogen seems to be unco-ordinated in these intermediate complexes.

Presumably, the ammonium groups of these protonated species adopt an *exo* conformation in order to minimize the electrostatic repulsion with the tricharged cation. Deprotonation of these groups and subsequent interconversion to an *endo* conformation is, most likely, the process determining the final, very slow equilibration step.

Conclusion

The information obtained in this study for the complexation *in vitro* of Gd^{3+} with ligands based upon the macrocyclic structure of 1,4,7,10-tetraazacyclododecane, and containing pendant functionalities, evidences several aspects which may be useful for the preparation of gadolinium-containing MRI contrast agents. (*i*) More stable complexes are formed by the ligands bearing negatively charged functionalities, although the complex stability is not strictly connected with the overall ligand charge. (*ii*) The co-ordination selectivity of these ligands towards Gd^{3+} does not follow the trend of complex stability, principally due to the different ligand basicities, and inversion of selectivity between ligands may occur upon changing the solution pH. (*iii*) Conversely, the thermodynamic resistance of the studied Gd^{3+} complexes towards demetallation processes promoted by the principal components of blood plasma, such as Na^+, Ca^{2+}, Cu^{2+}, Zn^{2+}, phosphate and carbonate, at physiological pH 7.4, is consistent with the trend of complex stability. (*iv*) Complexation of Gd^{3+} by these ligands takes place through a multi-step process lasting several weeks, but most of the free energy gain associated with the attainment of the final, more stable, complex species is produced in the first minutes of the process.

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