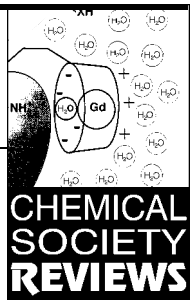
Lanthanide(III) chelates for NMR biomedical applications



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The peculiar magnetic properties of lanthanide(III) ions may be exploited for the development of powerful NMR probes for biomedical applications. Gd^{III} chelates are in current clinical use as contrast agents for magnetic resonance imaging. Other paramagnetic lanthanide(III) complexes endowed with shift reagent capabilities are used for the separation of NMR resonances of species present in the inner and outer cellular compartments and for the measurement of pH and temperature.

1 Introduction

In the last decade there has been a renewed interest in paramagnetic lanthanide(III) complexes because their peculiar magnetic properties have provided the route to tackle a number of problems in different fields of relevance to biomedicine by means of NMR techniques.[1]

The largest part of these studies has been devoted to Gd^{III} chelates used as contrast agents (CA) in conjunction with magnetic resonance imaging (MRI).[2,3] This is a powerful diagnostic technique which allows one to obtain images of tissues and organs which are topological representations of NMR parameters. Among these, longitudinal (R_1) and transverse (R_2) relaxation rates of water protons are the most important. The presence of paramagnetic Gd^{III} complexes causes a dramatic enhancement of the water proton relaxation rates and then allows one to add physiological information to the impressive anatomical resolution commonly obtained in the uncontrasted images. Thus, administration of Gd-based contrast agents has entered into the pool of diagnostic protocols and is particularly useful to assess organ perfusion and any abnormalities in the blood–brain barrier or in kidney clearance. Several other applications, primarily in the field of angiography and tumor targetting, are currently under intense scrutiny, with the promise of soon being available in clinical practice.

Besides Gd^{III} complexes, there is another important class of contrast agents for MRI which is based on polysaccharide-coated iron oxide particles. Their peculiarity stems from the fact that their blood half-life and distribution to different organs of the reticuloendothelial system (RES) depend upon the particle size. In general, larger particles are quickly sequestered by RES cells of the liver and spleen, whereas smaller particles remain in

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Silvio Aime was born in 1948 near Torino, Italy. He received the Laurea *degree from the University of Torino in 1971. Following a postdoctoral appointment at the University of East Anglia (with R. K. Harris) he returned in 1974 to Torino where he spent all his career. He is currently Professor of General and Inorganic Chemistry in the Faculty of Pharmacy. He is co-author of* ca. *250 papers and 4 patents in the field of organometallic chemistry, in particular the application of NMR spectroscopy to investigate solution and solid state properties of metal carbonyl clusters, and in bio-inorganic chemistry, with projects in the fields of relaxation and shift reagents for NMR applications in biomedicine, and on the role of metal ions in the etiology of Parkinson's disease.*

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the blood for a longer time and accumulate mainly in the lymph nodes.[4]

Nowadays about 35% of the MRI examinations make use of contrast agents, but this percentage is predicted to increase further following the development of more effective and specific contrast media than those currently commercially available.

Another major field of interest for the application in biomedicine of paramagnetic lanthanide chelates deals with their use as shift reagents to separate NMR signals of species present in the inner- and outer-cellular compartments.[5] The prototype application is the relative quantification of Na^+ and K^+ ions inside and outside red blood cells by using Dy^{III} or Tm^{III} complexes endowed with a high residual negative charge which does not allow them to cross the cellular membrane. It follows that the paramagnetic perturbation is confined to the extracellular environment and results in an alteration of the local magnetic field strength which in turn causes a significant shift in the resonance frequency of Na^+ or K^+ ions present in this compartment. In principle, this approach may be extended to other cationic, anionic and zwitterionic species provided there is the presence in the extracellular compartment of a shift reagent endowed with suitable electric charge distribution and molecular recognition properties. Furthermore, the resonances themselves of the paramagnetic shift reagents may be used as reporters of physico-chemical parameters like pH, temperature, *etc*.

In general, although no protocol for the *in vivo* use of shift reagents for humans has been approved yet, it is likely that the increasing availability of MRI instruments at 1.5 T (or more) incorporating high resolution NMR capabilities will increase the attention towards procedures able to exploit fully the diagnostic potential of these agents.

2 Gd^{III} complexes as contrast agents for MRI

The use of paramagnetic substances for increasing and controlling the magnetic relaxation of water protons has found wide application in the NMR techniques for medical imaging and diagnosis.[6]

The attention has been primarily focused on complexes of Gd^{III} since this metal ion with a *S* ground state electronic structure couples a large magnetic moment with a long electron spin relaxation time ($\sim 10^{-9}$ s at the magnetic field strengths of interest for MRI applications), two properties that ensure an optimum efficiency for nuclear spin relaxation of the interacting nuclei. Other general requirements of CA for MRI are low toxicity, rapid excretion after administration, good water solubility and low osmotic potential of the solutions clinically used. Moreover, since the free metal ions are poorly tolerated, they must be coordinated by a strongly binding ligand that occupies most of the available coordination sites. Eventually, the preferred metal complexes, in addition to showing high thermodynamic (and possibly kinetic) stability, should present at least one water molecule in their inner coordination sphere, in rapid exchange with the bulk solvent, in order to affect strongly the relaxation of all solvent protons.

The anionic complexes $Gd(DTPA)^{2-}$ (MAGNEVIST) and $Gd(DOTA)^-$ (DOTAREM) were the first complexes entered into clinical practice and they represent the reference compounds for the development and the evaluation of new agents. Later, two neutral complexes, Gd(DTPA–BMA) (OMNISCAN) and GdHPDO3A (PROHANCE), have been introduced with the aim of providing systems with reduced osmotic potential for applications requiring higher doses of CA.

Fig. 1 reports the schematic structure of the four ligands and the thermodynamic stability constants of their Gd^{III} complexes measured at 25 °C and $\mu = 0.1$. It may be surprising to find out that a complex like Gd(DTPA–BMA), whose stability constant is more than five orders of magnitude lower than $Gd(DTPA)^{2-}$, is considered safe enough for its clinical use. However, it has been pointed out that the toxicity[7] *in vivo* of Gd^{III} complexes with polyaminocarboxylate ligands does not necessarily correlate to the overall thermodynamic stability. Rather, the lack of displacement of Gd^{III} by endogenous Ca^{II} and the selectivity towards Ln^{III} ions introduced by two amide substituents are effective in setting off the net loss in the overall thermodynamic stability.

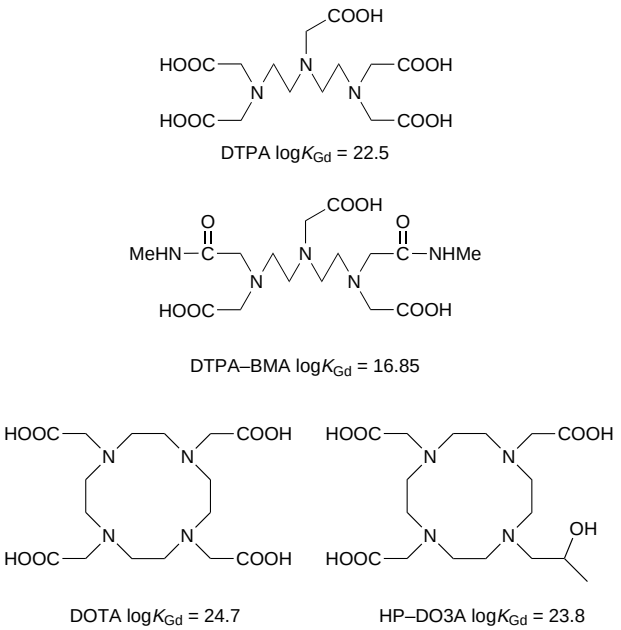

Fig. 1 Schematic representation of the four ligands whose Gd^{III} chelates are currently used as CA for MRI. Thermodynamic stabilities of the complexes at 25 °C and $\mu = 0.1$ M are reported.

An important step in the design and characterization of more effective CA is represented by the investigation of the relationships between the chemical structure and the factors determining their ability to enhance the water protons relaxation rates.

In the last few years we have investigated many of these complexes, by combining relaxometric and high resolution NMR techniques (on related complexes with $Ln \neq Gd$) and here we summarize the most relevant results.

2.1 Structural and dynamic determinants of the relaxivity of Gd^{III} complexes

The observed water proton longitudinal relaxation rate in a solution containing a paramagnetic metal complex is given by the sum of three contributions:[1,2,6]

$$R_1^{obs} = R_{1p}^{is} + R_{1p}^{os} + R_1^{w} \quad (1)$$

where R_1^w is the water relaxation rate in the absence of the paramagnetic compound, R_{1p}^{is} represents the contribution due to exchange of water molecules from the inner coordination sphere of the metal ion to the bulk water and R_{1p}^{os} is the contribution of solvent molecules diffusing in the outer coordination sphere of the paramagnetic center. The overall paramagnetic relaxation enhancement ($R_{1p}^{is} + R_{1p}^{os}$) referred to by a 1 mM concentration of a given Gd^{III} chelate is called its relaxivity.

A schematic representation of these two relaxation mechanisms operating the relaxation enhancement of the solvent water protons in solution by Gd^{III} complexes is shown in Fig. 2. The inner sphere relaxation rate is described in terms of the following set of equations:[1,2,8]

$$R_{1p}^{is} = \frac{c \times q}{55.6} \times \frac{1}{T_{1M}^{H} + \tau_M} \quad (2)$$

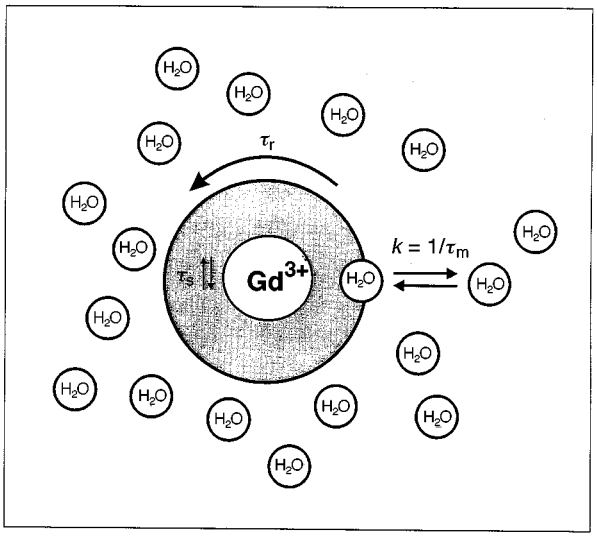

Fig. 2 Schematic view of the relaxation mechanisms (and the main relaxation parameters) operating in a aqueous solution containing a paramagnetic Gd^{III} chelate

$$\frac{1}{T_{1M}} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\hbar^2\gamma_S^2\gamma_H^2}{r_{GdH}^6} S(S+1)\left[\frac{3\tau_{cl}}{1+\omega_H^2\tau_{cl}^2} + \frac{7\tau_{c2}}{1+\omega_S^2\tau_{c2}^2}\right] \quad (3)$$

$$\frac{1}{\tau_{ci}} = \frac{1}{\tau_M} + \frac{1}{\tau_R} + \frac{1}{\tau_{Si}} \quad i = 1,2 \quad (4)$$

$$\left(\frac{1}{\tau_{S1}}\right)^{ZFS} = \frac{12}{5}\Delta^2\tau_v\left(\frac{1}{1+\omega_S^2\tau_v^2} + \frac{4}{1+4\omega_S^2\tau_v^2}\right) \quad (5)$$

$$\left(\frac{1}{\tau_{S2}}\right)^{ZFS} = \frac{12}{10}\Delta^2\tau_v\left(3 + \frac{5}{1+\omega_S^2\tau_v^2} + \frac{2}{1+4\omega_S^2\tau_v^2}\right) \quad (6)$$

In eqns. 2–6 c is the molar concentration of the paramagnetic complex; q is the number of water molecules coordinated to the metal ion; τ_M is their mean residence lifetime; T_{1M} is their longitudinal relaxation time; S is the electron spin quantum number; γ_S and γ_H are the electron and the proton nuclear magnetogyric ratios, respectively, r_{Gd-H} is the distance between the metal ion and the protons of the coordinated water molecules; ω_H and ω_S are the proton and electron Larmor frequencies, respectively; τ_R is the reorientational correlation time; and τ_{S1} and τ_{S2} are the longitudinal and transverse electron spin relaxation times. These last two are frequency dependent, according to eqns. 5 and 6, and characterized by the correlation time (τ_v) of the modulation of the transient zero-field splitting (expressed by the square of its trace value, Δ^2).

The outer-sphere relaxivity, which depends on the electronic relaxation time of the metal ion, on the distance of closest approach of solute and solvent (a) and on the sum of solvent and solute diffusion coefficients (D) is usually treated on the base of the set of equations developed by Freed.[9]

For small-sized complexes with $q = 1$ [such as $Gd(DOTA)^-$ and $Gd(DTPA)^{2-}$], it makes a contribution of roughly 40–50% to the observed relaxivity that, at high-field (> 10 MHz), is about the same for complexes of similar size and molecular weight. The solvent proton relaxation rate has a magnetic field dependence through eqns. 3–6 (and through the outer sphere equations as well) and thus the set of parameters involved in the paramagnetic relaxation theory can be best obtained through a magnetic field dependent study. Experimentally, this is performed by measuring solvent longitudinal relaxation rates over a wide range of magnetic fields with a field-cycling spectrometer that rapidly switches magnetic field strength over a range corresponding to proton Larmor frequencies of 0.01–50 MHz.[2] The data points represent the so-called nuclear magnetic relaxation dispersion (NMRD) profile that can be adequately fitted to yield the values of the relaxation parameters.

On dealing with multi-parameter equations it is desirable in the fitting procedure to fix the values of the parameters that can be determined through independent experiments. For instance, the q value may be obtained from luminescence studies of the corresponding Eu^{III} and Tb^{III} complexes, τ_R may be estimated from ^{13}C-T_1 measurement of a suitable C–H fragment in the related diamagnetic Y^{III}, La^{III} or Lu^{III} chelates, τ_M may be assesed (*vide infra*) from variable temperature measurements of the ^{17}O NMR transverse relaxation rate.

The inner sphere relaxivity in the high magnetic field region is mainly controlled by the reorientational correlation time, τ_R, which mainly depends upon the molecular dimension of the complexes, as shown by the good correlation between relaxivity and molecular weight for a number of structurally similar complexes. The low-field region of the NMRD profiles substantially differs among different complexes according to the zero-field value of their electronic relaxation time (τ_{S0}). The latter parameter [which may be easily calculated from Δ^2 and τ_v values through $\tau_{S0} = (12\Delta^2\ \tau_v)^{-1}$] is highly sensitive to the symmetry of the complex and to the chemical nature of the coordinating groups. In Fig. 3 the NMRD profiles of $Gd(DOTA)^-$ and $Gd(DTPA)^{2-}$ are reported. Since both complexes have one coordinated water molecule and very similar size and molecular weight, their relaxivities at high fields, which depend on $q\tau_R/r^6_{Gd-H}$, are also very similar. However, the two profiles differ considerably in the low field region as a consequence of their different electronic relaxation times. The axially symmetric $Gd(DOTA)^-$ complex has a τ_{S0} value higher than 700 ps, while for $Gd(DTPA)^{2-}$ this parameter assumes the value of 80 ps.

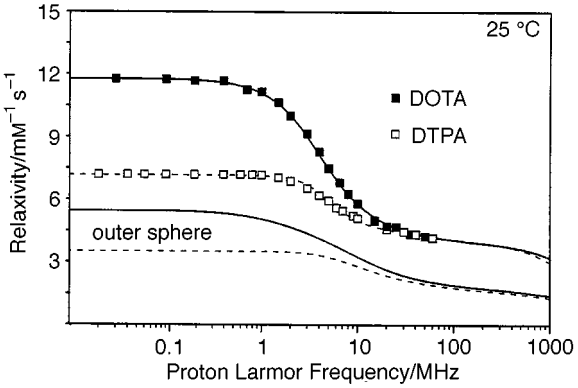

Fig. 3 $1/T_1$ NMRD profiles of $Gd(DTPA)^{2-}$ and $Gd(DOTA)^-$ at 25 °C. The lower curves represent the outer sphere contribution to the profiles.

Currently, the search for new CA for MRI is mainly directed toward the synthesis of Gd^{III} complexes of functionalized derivatives of DTPA and DOTA ligands without altering their chelating abilities. We studied a number of new complexes derived from the macrocyclic structure of DOTA by introducing one or more β-benzyloxy-α-propionic residues (Fig. 4).

These Gd^{III} chelates have been designed to interact, through the aromatic groups, with hydrophobic sites in biological molecules in order to improve their relaxivity (*vide infra*) and to increase their lifetime in the circulating blood. The NMRD profiles of the complexes are very sensitive to the chemical

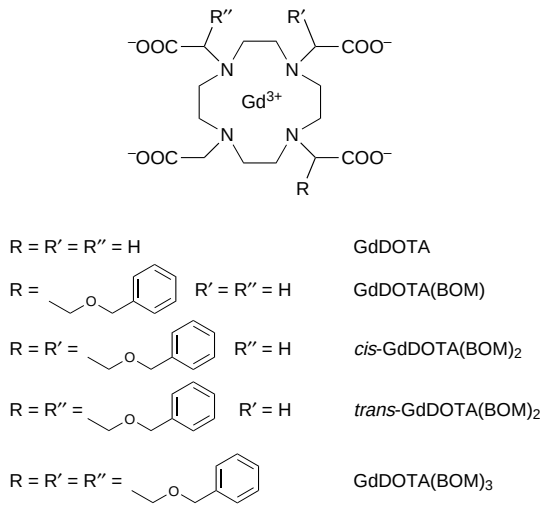

Fig. 4 Gd(DOTA)$^-$-like complexes bearing the β-benzyloxy-α-propionic residues (BOM)

modification of the chelate basic structure as shown in Figs. 5 and 6. All four Gd^{III} complexes have significantly higher relaxivities than Gd(DOTA)$^-$ over the entire magnetic field range investigated.

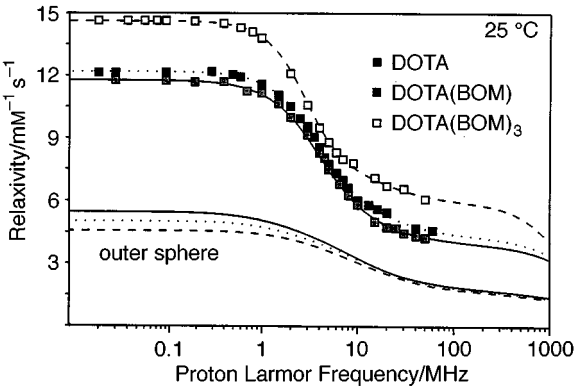

Fig. 5 Comparison between the $1/T_1$ NMRD profiles for Gd(DOTA)$^-$, Gd(DOTA–BOM)$^-$ and Gd(DOTA–BOM$_3$)$^-$ complexes at 25 °C. The lower curves represent the outer sphere contribution to the profiles.

The differences in relaxivity among the chelates are due to their different values of τ_R and τ_{S0}. At high fields the relaxivities show an almost linear dependence on τ_R which in turn is strictly related to the molecular weight and the size of the complexes, whereas at lower fields the relaxivity differences are well accounted for by the different values of τ_R and τ_{S0}.[10] The effect of the latter parameter is particularly evident when the relaxivity profiles of the disubstituted isomeric complexes are compared (Fig. 6). In this case the low field differences in the inner and outer sphere relaxivities are completely accounted for by the different electronic relaxation times of the two chelates. The value of τ_{S0} seems to reflect the changes in symmetry introduced in the coordination sphere of the Gd^{III} ion by the insertion of one, two or three β-benzyloxy-α-propionic residues. In fact, τ_{S0} of the mono-substituted complex (417 ps) is lower than that of the highly symmetric DOTA-complex. Moreover, the difference in τ_{S0} between the Gd^{III} complexes of *cis* (275 ps) and *trans* (443 ps) disubstituted ligands is particularly impressive and may result from the lower symmetry of the 1,4-disubstituted isomer. The value of τ_{S0} depends not only on the change introduced in the molecular geometry but also on the nature of the substituent group. In fact, the amidation of a carboxy group produces a dramatic decrease in τ_{S0}, which results in a lower water proton relaxivity at low fields. Moreover, the data obtained on a series of monoamide

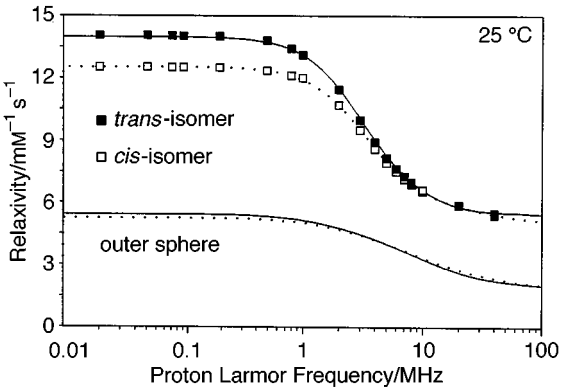

Fig. 6 $1/T_1$ NMRD profiles for the two isomers of Gd(DOTA–BOM$_2$)$^-$ at 25 °C. The lower curves represent the outer sphere contribution to the profiles.

derivatives of DOTA indicate that τ_{S0} is almost independent of the nature of the amide substituent (120–140 ps).[11] It is likely that the observed decrease in this parameter depends on the decreased donor ability of the amide oxygen with respect to the carboxylate oxygen. Therefore, the τ_{S0} parameter acts as a molecular amplifier of the minor differences in the coordination between the carboxylate and the carboxamide groups.

2.2 The role of the exchange lifetime τ_M

Although this parameter may affect the observed relaxivity either through eqn. 2 or eqn. 4, only recently has it been realized[12–14] that its value may be long enough to represent a limiting factor to the attainable relaxation enhancement promoted by Gd^{III} complexes. Graphical simulations through eqn. 2–6 (Fig. 7) show that an optimum value for this parameter,

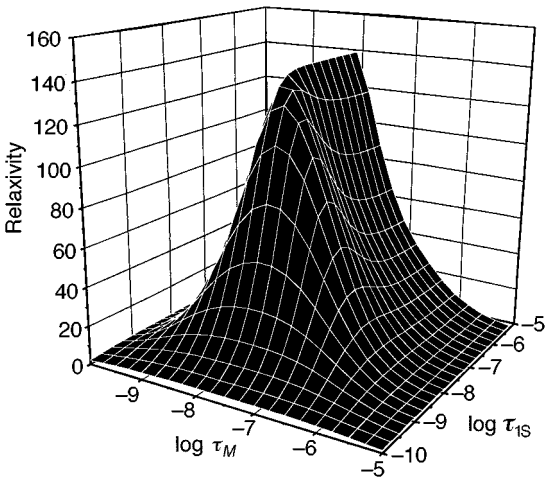

Fig. 7 3D representation of the τ_M and τ_S dependence of the longitudinal relaxivity for an immobilized (τ_R = 30 ns) Gd^{III} chelate (q = 1, r = 3 Å)

when the Gd^{III} chelate is bound to a slowly tumbling substrate like a protein, is in the range of few tenths of nanoseconds. An accurate determination of the exchange lifetime of the coordinated water molecule is pursued through the measurement of the transverse ^{17}O NMR relaxation time at variable temperature. The observed relaxation rates are dominated by the contact interaction and it is dependent either on τ_M or $\Delta\omega_M^O$ (which is the ^{17}O chemical shift difference between coordinated and bulk water) (eqns. 7–10):[13,14]

$$R_{2p}^O = P_M \tau_M^{-1} \frac{R_{2M}^{O\,2} + \tau_M^{-1} R_{2M}^O + \Delta\omega_M^{O^2}}{\left(R_{2M}^O + \tau_M^{-1}\right)^2 + \Delta\omega_M^{O^2}} \qquad (7)$$

$$R_{2M}^{O} = \frac{1}{3}\left(\frac{A}{\hbar}\right)^2 S(S+1)\left(\tau_{E1} + \frac{\tau_{E2}}{1+\omega_s^2\tau_{E2}^2}\right) \quad (8)$$

$$\tau_{Ei}^{-1} = \tau_{Si}^{-1} + \tau_M^{-1} \quad (9)$$

$$\tau_{Mj}^{-1} = \frac{\left(\tau_M^{-1}\right)^{298,15} T}{298.15} \exp\left[\frac{\Delta H_M}{R}\left(\frac{1}{298.15} - \frac{1}{T}\right)\right] \quad (10)$$

where (A/h) is the Gd–^{17}O scalar coupling constant (3.8×10^6 rad s^{-1} for the polyaminocarboxylate Gd^{III} chelate with only one metal bound water molecule), τ_{Ei} represent the correlation times of the processes modulating the scalar interaction, and ΔH_M is the activation enthalpy for the exchange process.

R_{2p}^{O} values increase as the temperature increases until τ_M becomes short enough with respect to R_{2M}^{O} thus causing a decrease of R_{2p}^{O} with a further increase of temperature. It is worth noting that R_{2M}^{O} is significantly higher than R_{1M}^{H} making this method much more sensitive to τ_M values with respect to the measurement of the longitudinal water proton relaxation rate. The resulting bell-shaped behaviour [Fig. 8 shows the profile

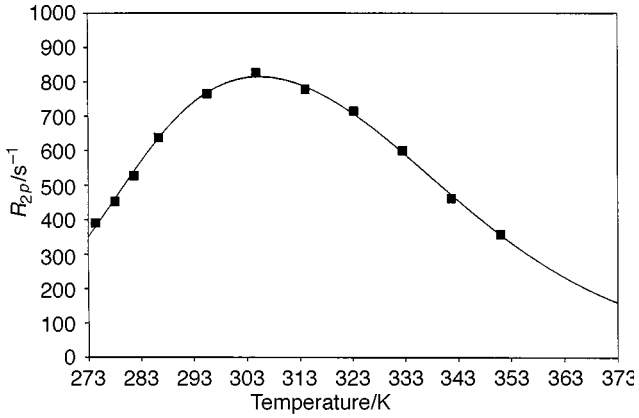

Fig. 8 Temperature dependence of the paramagnetic contribution to the water ^{17}O NMR transverse relaxation rate (R_{2p}) for Gd(DOTA)$^-$ (0.05 M) at 2.1 *T*

obtained for Gd(DOTA)$^-$] may be fitted through the above eqns. 7–10 affording the activation energy for the exchange process and the actual τ_M value at any temperature. From the data now available (Table 1) it is evident that τ_M (at 298 K) in Gd^{III} complexes may fall over an extended range of values, from a few nanoseconds to few microseconds. A simple, qualitative assessment of the occurrence of relatively long

Table 1 Water exchange lifetime at 25 °C for several Gd^{III} chelates as determined from the analysis of the temperature dependence of water ^{17}O transverse relaxation rate

Ligand	*q*	charge	τ_M/ns
aquo-ion	8	3+	1.2
DTPA	1	2−	303
DOTA	1	1−	244
DOTA(BOM)$_3$	1	1−	77
HP-DO3A	1	0	350
BMA–DTPA	1	0	2200
DOTMA	1	1−	68
DTMA[a]	1	3+	19000

[a] DTMA = 1,4,7,10,tetrakis-[(N-methylcarbamoyl)methyl]-1,4,7,10-tetraazacyclododecane.

exchange lifetime (> 0.5 μs) of the coordinated water molecule may also be drawn by measuring the proton relaxivity at temperatures lower than 25 °C; the flattening of the resulting profile of R_{1p}^{H} *versus T* at low temperature is a clear indication of the 'quenching' effect of the exchange lifetime (eqn. 2).[12] Inspection of the data reported in Table 1 indicates that, for systems with $q = 1$, the exchange is primarily determined by the residual electric charge and the structural properties of the complex. Now, since the exchange mechanism is dissociative, the exchange rate is determined by the difference in energy (ΔE) between the nine-coordinated ground state and the eight-coordinated activated state. On DOTA-like structures, the introduction of substituents on the square-antiprismatic coordination cage causes a decrease in the stability of the ground state (with respect to the parent DOTA complex) which results in a decrease of ΔE and, in turn, in a shortening of τ_M. Furthermore, Powell *et al.* have shown that a direct insight into the exchange mechanism of the coordinated water may be gained through ^{17}O NMR experiments at variable pressure.[15]

Interestingly, the occurrence of a long exchange lifetime of the coordinated water molecule in some of these paramagnetic chelates allows us to determine the contribution to the overall relaxivity arising from the exchange of protons only.[16,17] Let us consider the Gd^{III} complex of the bisbenzylamide–DTPA ligand, analogous to Gd(DTPA–BBA), and look at the pH dependence of the longitudinal water proton relaxation rate at room temperature and 20 MHz (Fig. 9). The higher relaxivity observed at basic pH arises then from the contribution of the base catalyzed prototropic exchange to the relatively slow exchange of the whole water molecule. Thus, the fast prototropic exchange at pH 12 removes the 'quenching' effect of the long τ_M and the R_{1p}^{H} value measured at this pH is then the expected value for this complex on the basis of its molecular size owing to the linear dependence of R_{1p}^{H} *versus* the molecular weight. Although not straightforward, one might envisage the possibility to shorten τ_M by increasing the prototropic exchange at physiological pH through the introduction of functionalities with suitable pK_a values in the close proximity of the coordinated water molecule.

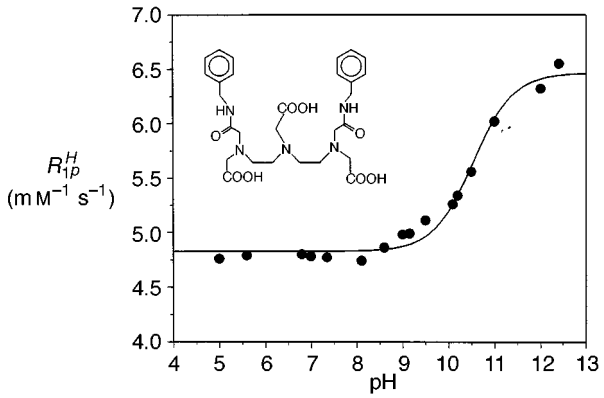

Fig. 9 pH dependence of the water proton longitudinal relaxivity (R_{1P}^{H}) for Gd(DTPA–BBA) at 20 MHz and 25 °C

2.3 Long τ_R values for enhanced relaxivities

At the magnetic field strengths currently employed in MRI (0.5–1.5 T, corresponding to proton Larmor frequencies of 20–60 MHz), the ability of Gd^{III} chelates to enhance the longitudinal water proton relaxation rate is mainly determined by the value of their molecular reorientational time, τ_R. Therefore, the achievement of higher water proton relaxation rates may be pursued through an increase of this parameter, since the increase of the number of the metal bound water molecules (q), which would lead to the same result, is likely accompanied by a decrease of the stability of the complex. It has been shown that the effectiveness of Gd^{III} complexes as CA may be significantly improved by using protein–chelate

conjugates in which the metal complex is covalently attached to amino acid residues of the protein; this approach, then, couples the strong chelation of the metal ion with the slow molecular tumbling of the protein.[18] However, severe limitations may arise from a poorly controlled addition of the ligand molecules to the protein, which may result in a decreased thermodynamic stability of the chelate and in an alteration of the hydrophobic–hydrophilic domains of the macromolecule. An alternative route for increasing τ_R may be pursued through the formation of host-guest non-covalent interactions between suitably functionalized complexes and slowly tumbling macromolecules.[19] We have investigated the non-covalent interactions between a variety of substituted derivatives of $Gd(DTPA)^{2-}$ and $Gd(DOTA)^{-}$ and put forward a general picture accounting for the main determinants of the relaxation enhancement observed when a paramagnetic Gd^{III} complex is bound to human serum albumin (HSA).[20]

In addition to providing high relaxivities (which, in turn, allows one to reduce the administered doses of CA) the formation of adducts between Gd^{III} complexes and HSA are of notable interest for the design of novel angiographic experiments for which an increased residence time and a better compartmentalisation in the circulating blood is required. As schematically shown in Fig. 10, we have shown that the observed relaxation enhancement in these systems receives a substantial contribution also from water molecules in the hydration shell of the macromolecule and protein exchangeable protons which lie close to the interaction site of the paramagnetic complex.

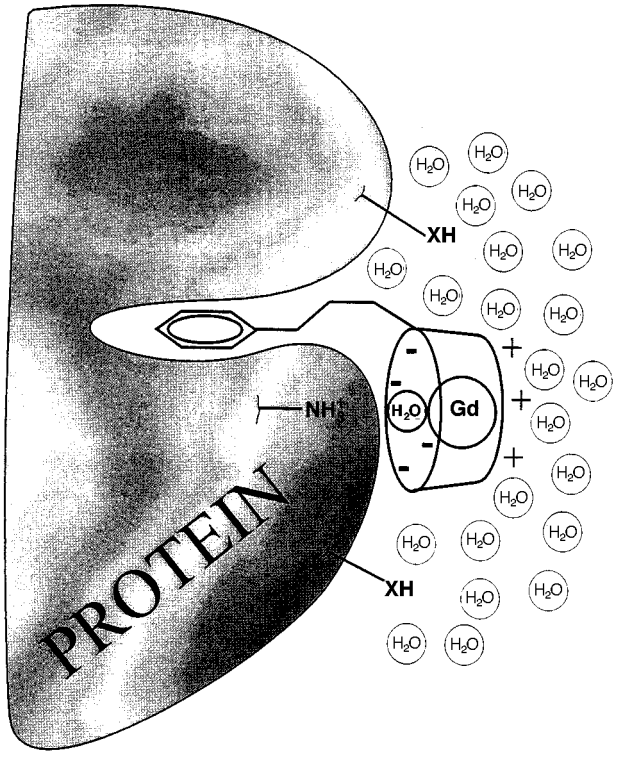

Fig. 10 Schematic representation of the non-covalent interaction between a protein and a Gd^{III} chelate bearing an hydrophobic residue

According to the type of substituent, the thermodynamic association constant of these complexes with HSA varies from 10^2 to 10^4 M^{-1} whereas the number of binding sites has been found to vary from 1 to 3. Competition assays performed with suitable probes allow us to map the interaction sites. For instance, we found that the Gd^{III} complex of a DOTA-like ligand bearing three β-benzyloxy-α-propionic substituents displays two equivalent binding sites on HSA located in subdomains IIA and IIIA of the protein. The macromolecular adduct resulting from the interaction of this complex with HSA has a relaxivity of about 56 mM^{-1} s^{-1} (at 39 °C and 20 MHz), which represents the highest value so far reported for a Gd^{III} chelate.[20] NMRD profiles are highly diagnostic of the formation of adducts between Gd^{III} complexes and slowly moving substrates like proteins as they show a relaxivity peak centered at about 20–30 MHz (Fig. 11). Indeed, slowly rotating systems are characterised by a τ_R value which is too long to contribute to τ_C at low fields, where τ_S is short. Since the actual τ_S value increases with frequency (eqn. 5), it becomes longer than τ_R and τ_C goes from τ_S to τ_R at a frequency corresponding to τ_S^{-1}. At higher frequency the conditions $\omega_H\tau_R \geqslant 1$ occurs.

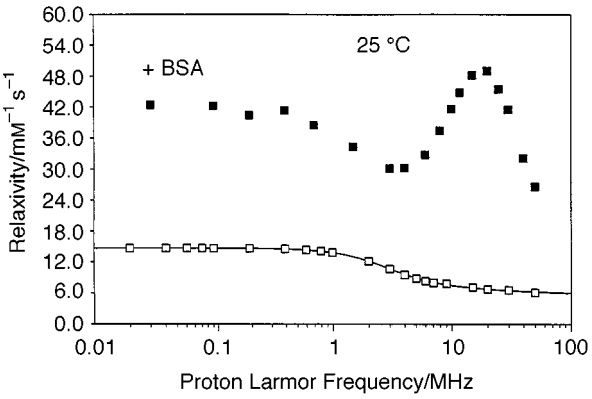

Fig. 11 $1/T_1$ NMRD profiles for $Gd(DOTA\text{–}BOM_3)^{-}$ with (■) and without (□) bovine serum albumin (25 °C, pH 6.9)

2.4 Relaxation enhancement in systems with $q = 0$

As mentioned before, when a Gd^{III} complex does not possess any water molecule in its inner coordination sphere, the enhancement of the solvent relaxivity is exclusively due to the electron–nucleus dipolar interaction between the metal ion and the water molecules diffusing in the outer coordination sphere of the complex. This interaction is modulated by the translational diffusional motion of solute and solvent and by the electronic relaxation time. This mechanism makes a contribution of roughly 40–50% to the overall relaxation rate for low molecular weight Gd^{III} chelates with octadentate ligands and $q = 1$[1,2] and, in principle, it could be evaluated experimentally by considering systems with $q = 0$. However, because of the preference of Gd^{III} for a coordination number of 9, this situation is rather uncommon. Recently, we have studied a highly rigid, kinetically stable, 8-coordinate Gd^{III} complex of a macrocyclic benzylphosphinate ligand (BzDOTP, Fig. 12).[21] In this case the

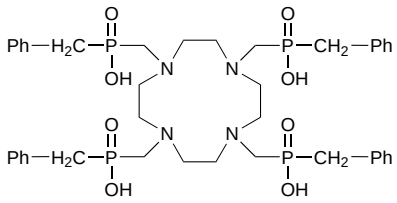

Fig. 12 Scheme representing the BzDOTP ligand

magnitude of the relaxivity and the nature and form of the NMRD profile are fully consistent with the behaviour of a complex which does not possess any contribution from a bound water molecule. The analysis of the profile suggests that the nearest water molecule is 4.25 Å distant from Gd. This complex represents then a good example of a pure 'outer-sphere' contrast agent. Furthermore, $Gd(BzDOTP)^{-}$ has been found to form a relatively strong complex with bovine serum albumin (BSA; $K_A = 3.6 \times 10^3$ M^{-1} at 25 °C) leading to a marked (for a $q = 0$ Gd^{III} complex!) relaxivity enhancement (Fig. 13). In fact, a remarkably high efficacy of the complex in liver and bile has been observed in MRI examinations. A major contribution to

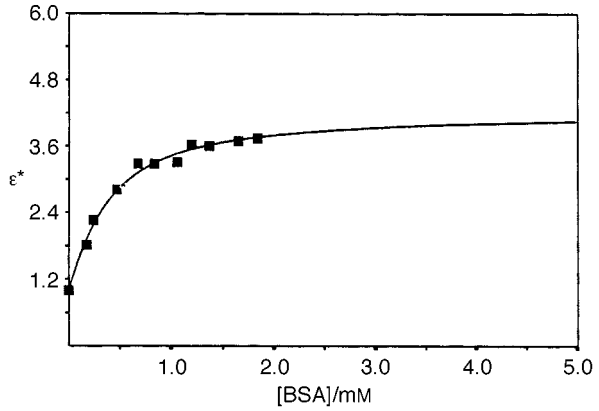

Fig. 13 Proton relaxation enhancement for Gd(BzDOTP)$^{-}$ (0.2 mM), at 20 MHz and 25 °C, as a function of bovine serum albumin concentration

the relaxivity enhancement is likely to be due to the exchange of the mobile protons of the protein which are dipolarly relaxed by the proximity to the paramagnetic center. Another possible contribution could arise from the high structural organisation and the consequent reduced mobility of the solvent molecules in the hydration sphere of the protein, near the binding site of the complex, which allows the observation of second-sphere interactions. This contribution to the overall relaxivity has been recently described in the case of complexes of 1,4,7,10-tetraazacyclododecane containing one carboxamide and three phosphinate substituents.[22] It has been found that the observed relaxation enhancement of water protons (Fig. 14) is determined, in addition to the expected outer-sphere mechanism, by a relatively distant water molecule in the second coordination sphere. This is explained by the participation of the amide carbonyl oxygen in hydrogen bonding to a local water molecule which results in a short enough metal–proton distance.

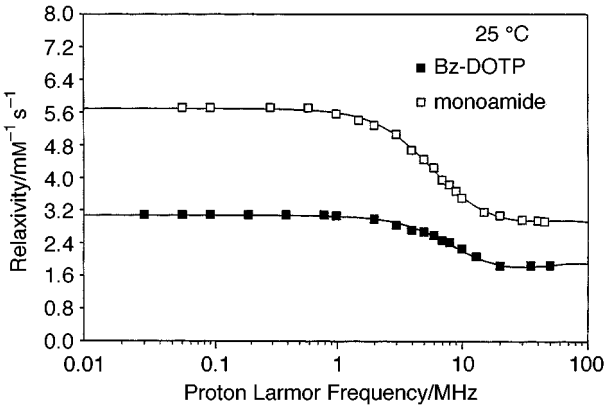

Fig. 14 $1/T_1$ NMRD profiles for Gd(BzDOTP)$^{-}$ and for a macrocyclic Gd^{III} chelate containing three methylphosphinate groups and one bis-*n*-buthylcarboxoamide group

In some cases, the outer sphere relaxivity may be so high as to induce an erroneous estimation of the *q* value. This happened in the case of Gd(DOTP)$^{5-}$ [DOTP = 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetrakis(methylenephosphonic acid)] which displays a relaxivity of 4.7 s^{-1} mM^{-1} at 25 °C and 20 MHz, a value quite reasonable for a system with $q = 1$ [*e.g.* Gd(DOTA)$^{-}$ has the same relaxivity under the same experimental conditions]. Later, ^{17}O NMR R_{2p} measurements unambigously showed that the complex has no water molecule directly coordinated to the paramagnetic center. Then, the high relaxivity appears determined by the ability of the phosphonate groups to form a strong second hydration sphere around the complex. These water molecules are in fast exchange with the bulk solvent and their contribution to the overall relaxivity may be evaluated by the same set of eqns. 2–6 above introduced for the assessment of the inner sphere term. Moreover, this complex displays a strong interaction with hemoglobin at the binding site of the natural 2,3-diphosphoglycerate allosteric effector.[23] Very interestingly, this makes the observed relaxivity dependent upon the conformational state of the protein.

2.5 Improved contrast agents

The search for a new generation of CA appears strongly orientated to provide them with high tissue and/or organ specificity. In this context, there is much interest to develop CA able to detect malignant focal lesions and to differentiate them from non-malignant ones. In particular, in order to target hepatocytes, the route to exploit, among various possibilities, the known properties of the transport system of the baso-lateral membranes has been pursued.[24] Accordingly, a Gd^{III} complex is expected to enter the hepatocytes if it bears, on the surface of the ligand, a synthon already known to be recognized by the transport system of the hepatocyte. Schematically, these CA are formed by three components: the recognition synthon, a spacer and the Gd^{III} containing moiety (Fig. 15).

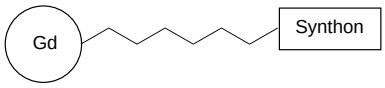

Fig. 15

As recognition synthons either bile acid residues or iodinated species (namely 3-amino-2,4,6-triiodobenzoic acid and iopanoic acid) have been used. Both synthons are particularly able to enter the hepatocytes as well documented in studies of related hepatospecific X-ray CA. By this approach, the hepatobiliary excretion of DTPA- and DOTA-like systems may reach values as high as 50–60%. It is worth mentioning that the highly hydrophilic parent Gd(DTPA)$^{2-}$ and Gd(DOTA)$^{-}$ have an almost 100% renal excretion. Animal biodistribution of a given contrast agent is usually assessed, in addition to the direct MRI evaluation, by means of the investigation with radioactive ^{153}Sm and ^{159}Gd chelates.[25]

3 Applications of Gd^{III} complexes to *in vitro* quantitative assays

A feedback of the research efforts to afford new CA for MRI has provided a new route to *in vitro* quantitative determination of a number of species through the observation of the effects on the relaxation properties of solvent water protons caused by the interaction that suitable paramagnetic complexes are able to set up with the analytes to be determined. As a first test to probe this idea we chose the determination of glycated albumin which may be present in significant amount in blood serum in the presence of high glucose levels.[26] The non-enzymatic glycation of proteins is a quite common process, and it is known to involve mainly ε-terminal amino groups of lysine residues. As a paramagnetic probe we dealt with a functionalized derivative of Gd(DTPA)$^{2-}$. To recognise the glycated protein we chose the boronic functionality, whose ability to form a stable ester bond with *syn*-diols is well established, and an affinity chromatography method based on boronic functionalised resin is currently used among others in clinical practice to determine glycated hemoglobin. The synthesis of the DTPA ligand functionalised with boronic acid was carried on by reacting DTPA anhydride with 3-aminophenylboronic acid. The obtained bisamide ligand was then reacted with an equimolar amount of $Gd_2(CO_3)_3$ to afford the corresponding Gd^{III} complex. In the presence of glycated proteins, the boronate on the complex forms a stable ester bond with the *syn*-diol of the protein sugar (Fig. 16). As a consequence, the reorientational correlation time of the complex will become longer and the water proton relaxation rate will increase significantly. In order to check the potential utility

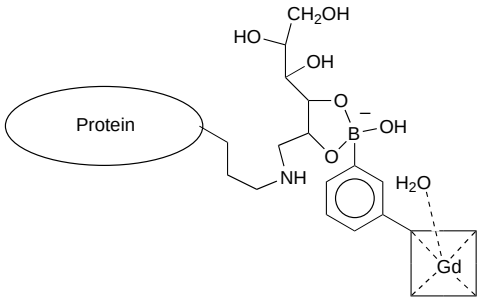

Fig. 16 Schematic representation of the covalent binding between the *syn*-diol group of a glycated protein and the Gd^{III} chelate functionalised with a boronic residue

of this approach to quantify the correct amounts of glycated protein in an actual specimen, we compared the proton relaxation rates of solutions of this Gd^{III} bisamide complex containing variable amounts of HSA at different degrees of glycation to the results obtained from the fructosamine method used in the clinical chemistry practice. The good linearity found between the observed relaxation rate and the protein sugar concentration in the albumin solutions (Fig. 17) is very promising to support such proton relaxation enhancement approaches as a new method for the determination of glycated proteins. More generally, we believe that these results introduce a novel area of development of paramagnetic Gd^{III} complexes in addition to their well established role as contrast agents for MRI. The use of functionalised paramagnetic complexes may allow the easy and quick determination of a variety of macromolecular substrates through the detection of the increase of solvent water proton relaxation rate as the result of the formation of slowly tumbling macromolecule–complex adducts. Interestingly, the proposed procedure represents the NMR counterpart of the EPR free radical assay technique based on the detection of changes in the linewidth of nitroxide resonance caused by the interaction of the labeled reagent with macromolecules.[27]

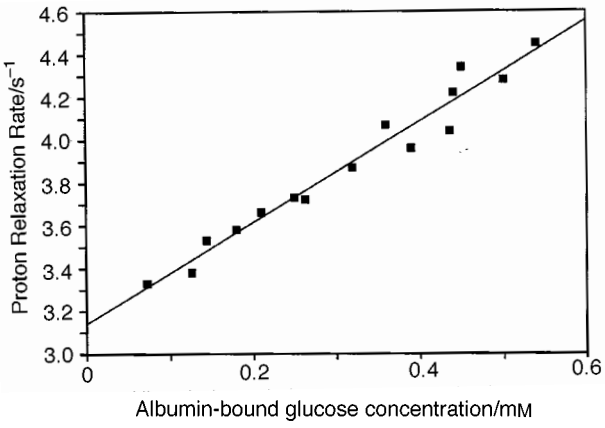

Fig. 17 Water proton relaxation rates of 0.56 mM solution of the Gd^{III} chelate bearing the boronic functionality as a function of the concentration of glycidic groups on human serum albumin as determined by the fructosamine method

Very recently, another interesting strategy for constructing relaxation reagents whose relaxivity is dependent upon the biochemical environment has been proposed. The prototypical example is represented by a Gd–DOTA like complex containing the galactopyranosyl ring bound to the tetraazamacrocycle in a way that prevents the coordination of the water molecule to the Gd^{III} ion. When this substituent is removed by the activity of the β-galactosidase enzyme, the access of water becomes allowed with a consequent increase of the observed relaxivity. These agents then generate distinct 'on' and 'off' states by controlling the access of water molecules to a chelated paramagnetic Gd^{III} ion.[28]

4 Ln^{III} chelates as shift reagents

4.1 Metal cations

NMR active metal cations of clinical importance such as $^{7}Li^{+}$, $^{23}Na^{+}$ and $^{39}K^{+}$ are normally found, in biological systems, in both the intra- and extra-cellular compartments and, in routine high resolution NMR spectra, are characterized by a single resonance. The application of metal NMR spectroscopy to biomedical studies implies obtaining distinct signals from the the two compartments that may then be simultaneously monitored and individually analyzed.[5] This is made possible by the use of aqueous shift reagents (SR), water soluble paramagnetic metal chelates which only distribute in the extracellular space and thus are able to remove the signal degeneracy by selectively affecting the extracellular resonance. On the basis of the experience gained over the last fifteen years by different laboratories, it has been established that, in order to be an effective SR, a metal chelate must satisfy several requirements and present certain well defined characteristics:

(a) The paramagnetic metal ion, in a non-*S* ground state electronic configuration, should present a high magnetic moment and a short value of the electronic relaxation time ($\sim 10^{-13}$ s). This requirement normally restricts the choice, among the lanthanide(III) ions, to Dy^{III}, Tb^{III}, and Tm^{III}.

(b) High negative charge. The efficacy of a SR (*i.e.* its ability to produce a large shift of the cation resonance in the extracellular compartment) is strictly related to the possibility of favouring multiple strong electrostatic interactions (ion-pairs) with the metal cations. Obviously the binding requires negatively charged groups on the surface of the coordination cage (carboxylic, phosphonic, *etc.*), either coordinated to the paramagnetic centre or pendant. Furthermore the overall negative charge of the SR does not allow the crossing of the phospholipidic membranes and thus favours their distribution in the extracellular space. Unlike T_1-water relaxation reagents described above, the cations to be monitored do not enter the first coordination sphere of the paramagnetic metal ion and therefore all the coordination sites may be occupied by the donor atoms of the ligand.

(c) The extent of the shift effect also has a dependence from a term of the type $(3\cos^2\theta - 1)/r^3$, where r and θ are the polar coordinates of the nucleus under study with respect to the Ln^{III} ion and with the main magnetic axis. It follows that the effect will be maximum for axially symmetric complexes having the cation binding sites along the symmetry axis. Moreover, due to the dependence of the shift on the $1/r^3$ term, the complexes bearing the negative charge on unbound side chains are expected to be less effective SR. Up to now in laboratory practice, four complexes have been studied in detail and applied to the answering of several questions of biomedical interest:[5] the Dy^{III} complexes of PPP^{5-} (triphosphate) and $TTHA^{6-}$ (triethylenetetraminehexaacetate) and the Dy^{III} and Tm^{III} complex of $DOTP^{8-}$.

The most effective SR so far reported is the chelate $Dy(PPP)_2^{7-}$, first introduced in 1982. The $Dy(TTHA)^{3-}$ complex is much less toxic as a consequence, probably, of the high stability constant of Dy^{3+} with this multidentate ligand. However, the reduced value of the complex negative charge and the fact that it is mainly localized on unbound carboxylic groups, away from the paramagnetic centre, make this metal chelate less effective in removing the signal degeneracy. The Dy^{III} and Tm^{III} complexes of DOTP represent an important improvement in the search for safer and more effective SR for metal cations of biological relevance (Fig. 18). In fact, these metal chelates are extremely resistant to dissociation processes over a wide range of pH, and present interaction sites for the cations very close to their four-fold axis of symmetry, thus ensuring a maximum shifting efficency. Actually, it has been

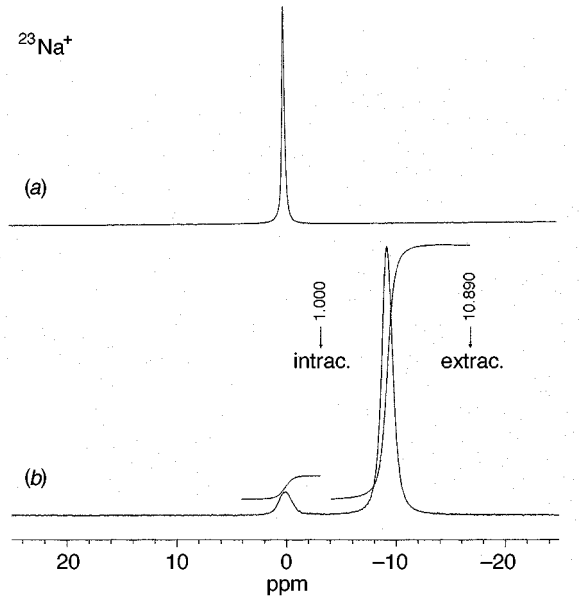

Fig. 18 ^{23}Na NMR spectra of human blood before (*a*) and after (*b*) the addition of 5 mM of Dy(DOTP)$^{5-}$ (39 °C at 2.1 *T*)

shown that TmDOTP^{4-} produces resolved ^{23}Na resonances in the *in vivo* rat liver with relatively little compromise in commonly measured physiological indices.[29] Moreover, its application to the *in vivo* rat kidney showed that this shift reagent produces three resolved resonances from intracellular Na^+, combined interstitial and vascular Na^+, and filtrate Na^+.[30]

In conclusion, it is worth commenting that, although several studies have been possible by the use of the available SR, much remains to be done in terms of synthetic strategy and ligand design in the search for safer and more effective compounds.

4.2 Anions

In principle, analogous considerations may be used for the development of Ln^{III} complexes acting as SR for anions like phosphate, carbonate, chloride, lactate, *etc.* In this context, we have considered the cationic macrocyclic Eu(DTMA)$^{3+}$ complex (DTMA = 1,4,7,10-tetrakis-[(*N*-methylcarbamoyl)methyl]-1,4,7,10-tetraazacyclododecane) as SR for the phosphate ion.[31] As shown in Fig. 19*a*, the ^{31}P NMR spectrum of human blood shows a single resonance for the inorganic phosphate ion flanking one of the two diphosphoglycerate (DPG) resonances. Upon addition of Eu(DTMA)$^{3+}$, a new signal is clearly detectable (Fig. 19*b*) which is assigned to the extracellular phosphate experiencing the additional field of the paramagnetic metal ion. Up to now, this represents the first tentative step in a field that is likely to receive much more attention in the near future.

5 Water signal suppression in ^{1}H NMR spectra of biological fluids promoted by Dy^{III} complexes

Solvent suppression in ^{1}H NMR spectra has received considerable attention in recent years in biochemical applications of NMR spectroscopy. Spectral selection methods have been proposed over the years, based on the differences between the longitudinal or transverse relaxation rates of water protons compared to those of other species present in solution. Among them, the WATR method (water attenuation by T_2 relaxation) introduced by Rabenstein *et al.*[32] is based on the increase of water transverse relaxation rates by chemical means, resulting in a selectively attenuated solvent signal in the spin-echo spectra. Chemicals which cause such a decrease of water T_2 relaxation times are species containing mobile protons which can exchange with the solvent water. Aqueous solution of Dy^{III} complexes with DOTA-like ligands can act as paramagnetic

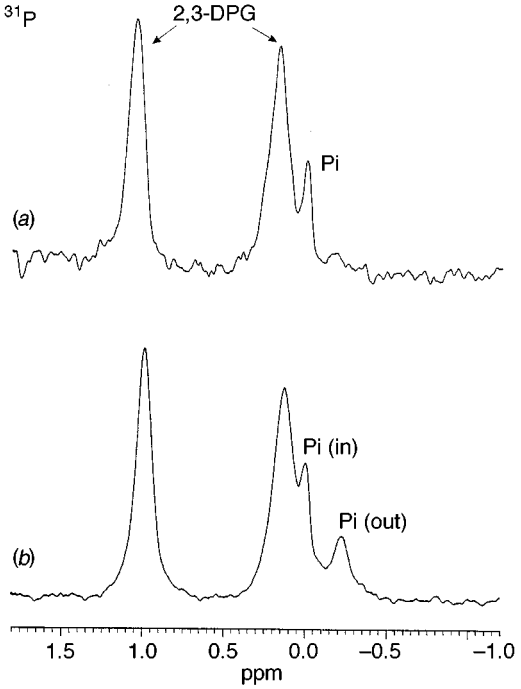

Fig. 19 31P NMR spectra of human blood before (*a*) and after (*b*) the addition of Eu(DTMA)$^+$ (39 °C at 2.1 *T*) (2,3-DPG = 2,3-diphosphoglycerate; Pi = inorganic phosphate)

WATR agents at low concentration and over a wide pH range and were tested in water signal suppression experiments by using the CPMG pulse sequence as suggested in the WATR method.[33] In principle, their ability to suppress the water signal depends upon the paramagnetic shift, the relaxation time and the exchange lifetime of the coordinated water protons. The pH of the solution (from 2 to 11) had no detectable effect on R_2. An illustrative spectrum obtained by the addition of a Dy^{III} DOTA-like complex (2.5 mM) to a normal human CSF (5% D_2O) is shown in Fig. 20. Typical resonances from glucose, lactate, acetate, citrate, creatinine, *etc.* are readily detected, in agreement with previously reported ^{1}H NMR spectra of CSF specimens. We have observed neither any detectable paramagnetic shift nor broadening of these resonances. This may be the result of the tight molecular geometry of the complex, which largely limits the access of organic substrates into the inner coordination sphere of the metal ion.[34] Furthermore, the proton resonances of the paramagnetic complex fall in a wide absorption range (over 200 ppm) but their very short T_2 values do not allow their detection in the spin-echo ^{1}H NMR spectra.

6 Paramagnetic Ln^{III} complexes as reporters of the physico-chemical environment

6.1 pH indicators

As just mentioned, a characteristic NMR property of paramagnetic Ln^{III} complexes is the large chemical shift range usually shown by ligand resonances. Thus, it is common that the ^{1}H resonances of these complexes usually fall outside the diamagnetic region and then do not overlap with those arising from endogenous diamagnetic molecules. Moreover, these paramagnetically shifted resonances are highly sensitive to slight structural and electronic variations. We found that these compounds may represent excellent NMR pH indicators provided that they contain acid/base functionalities whose pK_a values fall in the pH range of interest. As a representative example of this class of complexes we report the results obtained with Yb(DOTP)$^{5-}$.[35] As we have seen in a previous paragraph, the corresponding Dy(DOTP)$^{5-}$ and Tm(DOTP)$^{5-}$ chelates are used as shift reagent to differentiate extra- and intra-cellular signals of NMR-active cations. The ^{1}H NMR

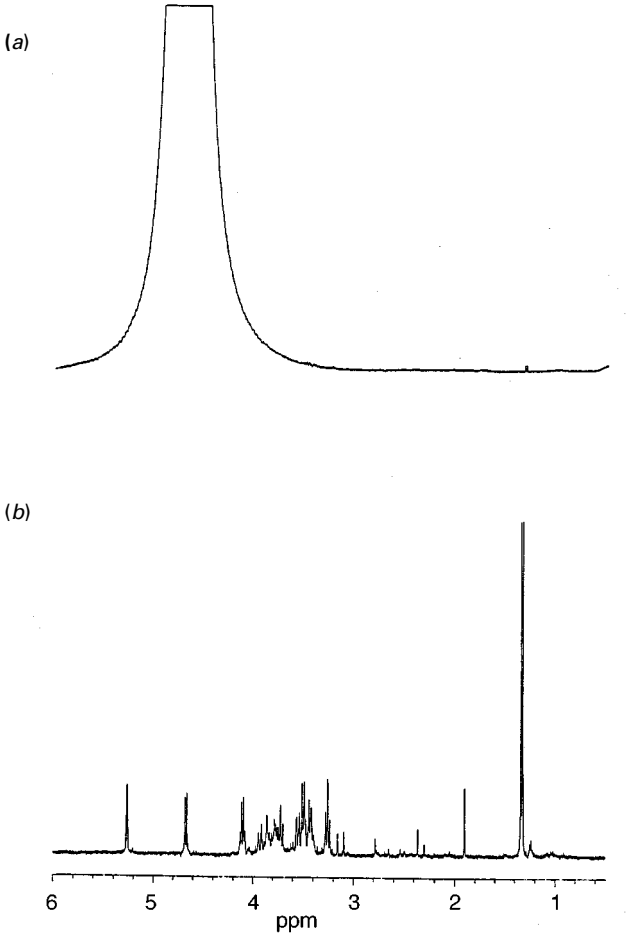

Fig. 20 ^{1}H-NMR spectra of normal human CSF in 95% H_2O–5% D_2O solution containing a 2.5 mM concentration of Dy^{III}-complex (25 °C at 9.4 *T*). (*a*) single pulse method. (*b*) Carr–Purcell–Meiboom–Gill spin-echo spectrum.

spectrum of $Yb(DOTP)^{5-}$ (Fig. 21) is consistent with a stereochemically rigid system of D_4-symmetry. At basic pH values there are five residual negative charges on the complex, located on the eight uncoordinated oxygen atoms of the phosphonate groups, which progressively decrease when the pH of the solution is lowered. The stepwise addition of H^+ ions causes large changes in the chemical shift of all the resonances

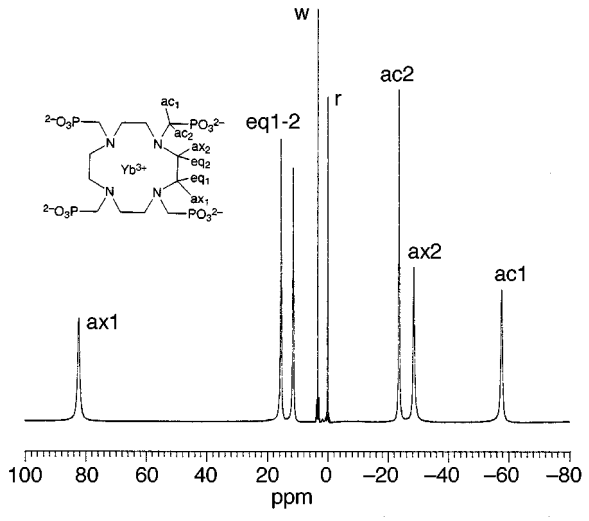

Fig. 21 ^{1}H NMR spectrum of a 25 mM solution of $Yb(DOTP)^{5-}$ at 39 °C, 2.1 *T* and pH 7.1 (w = HOD resonance; r = *tert*-butyl alcohol resonance used as internal reference, δ = 0 ppm)

whose values become then reporters of the pH of the solution. In order to avoid the use of an internal reference to quote the shift of the pH-dependent resonances, it is advantageous simply to consider the chemical shift separation between a selected pair of resonances. For instance, the dependence of $\delta_{ax1} - \delta_{ac1}$ upon pH appears to be linear in the pH range between 5.0 and 7.5, with a slope of 7.0 ± 0.1 ppm (pH unit)$^{-1}$.

6.2 Temperature sensitive probes

It is well known that the chemical shift of proton resonances in paramagnetic complexes is strongly temperature dependent. In the absence of specific interactions between the complex and endogenous substrates, one may safely assume that changes in chemical shift of the ligand resonances may act as a reporter of temperature changes in a given organ or tissue. For this application we chose $Yb(DOTMA)^-$ whose ^{1}H NMR spectrum approximately spans 170 ppm (Fig. 22).[36] Its fourfold symmetry axis reduces the number of ^{1}H resonances to six. Analogously to the approach described for the pH indicator, it is more advantageous simply to compare the chemical shift difference between a pair of resonances falling respectively at the low and high frequency side of the normal (diamagnetic) proton spectrum. In Fig. 23 the temperature dependence of the change in chemical shift difference between δ_{ac} and δ_{ax1} proton resonances (measured in human serum) between 35 °C and 45 °C is reported. A straight line fit of the experimental data points gave a slope of −0.41 ± 0.01 ppm °C^{-1}.

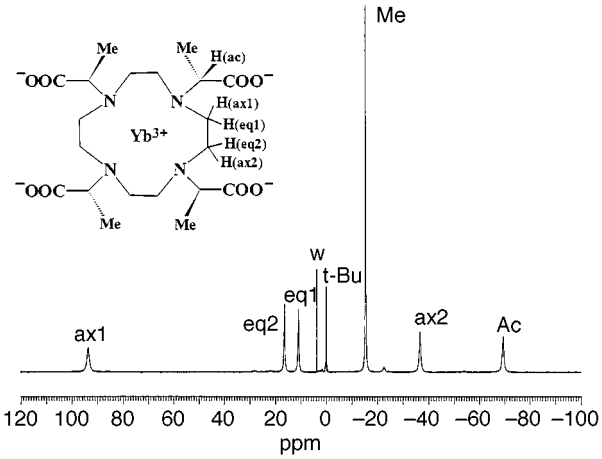

Fig. 22 ^{1}H-NMR spectrum of $Yb(DOTMA)^-$ at 27 °C, 2.1 *T* and pH 7.1 (w = HOD resonance; t-Bu = *tert*-butyl alcohol resonance used as internal reference, δ = 0 ppm)

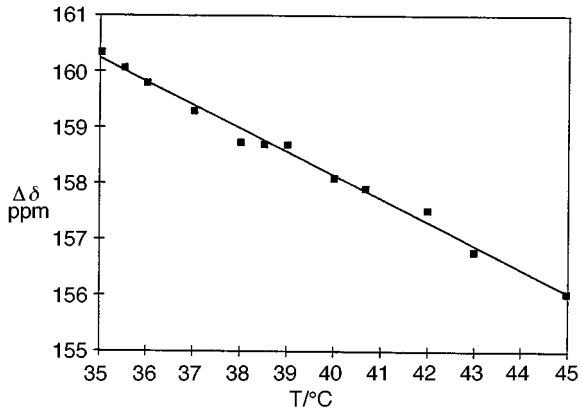

Fig. 23 Temperature dependence of ax1-ac proton chemical shift difference as measured in human serum containing 25 mM of $Yb(DOTMA)^-$

From this work we draw the suggestion that the interesting properties of the high resolution NMR spectra of paramagnetic Ln^{III} complexes (when Ln ≠ Gd) may find a novel application in MRI.[36] In fact, in principle, it may be possible to map the

spatial distribution of a paramagnetic complex provided a sufficiently intense signal with a chemical shift that is far enough from those of water and other tissue constituents is available. This has been shown for Yb(DOTMA)$^-$ by selectively exciting the most intense methyl group resonance (12 protons, −14.2 ppm at 27 °C) for complex concentration ranging from 0.003–0.1 M. A distinct advantage of dealing with paramagnetic complexes is that the protons in the complexes are expected to exhibit extremely short T_1 relaxation times, allowing very rapid acquisition times.

7 Concluding remarks

The maturation of magnetic resonance approaches to tackle *in vivo* biochemical problems as well as to improve the specificity of clinical investigations will prompt the development of novel lanthanide complexes in order to enhance the physiological information from this technique. The increased availability in hospitals of MRI instruments capable of providing spectroscopic in addition to morphologic information introduces stronger links between chemists, biochemists and physicians leading towards a molecular view of biological problems. This, in turn, will contribute to the design and use of suitable chemicals which allow a better understanding of complex systems. On the basis of the results herein reviewed, we believe that much innovative work may come from exploiting the peculiar magnetic properties of paramagnetic Ln^{III} complexes.

8 Acknowledgements

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