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Silvio Aime · Mauro Botta · Mauro Fasano
Simonetta Geninatti Crich · Enzo Terreno

Gd(III) complexes as contrast agents for magnetic resonance imaging: a proton relaxation enhancement study of the interaction with human serum albumin

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Abstract The non-covalent interaction between human serum albumin (HSA) and DOTA-like Gd(III) complexes containing hydrophobic benzyloxymethyl (BOM) substituents has been thoroughly investigated by measuring the solvent proton relaxation rates of their aqueous solutions. The binding association constants (K_A) to HSA are directly related to the number of hydrophobic substituents present on the surface of the complexes. Furthermore, an estimation of $\Delta H°$ and $\Delta S°$ has been obtained by the temperature dependence of K_A. Assays performed with the competitor probes warfarin and ibuprofen established that the complexes interact with HSA through two nearly equivalent binding sites located in the subdomains IIA and IIIA of the protein. Strong relaxation enhancements, promoted by the formation of slowly tumbling paramagnetic adducts, have been measured at 20 MHz for complexes containing two and three hydrophobic substituents. The macromolecular adduct with the latter species has a relaxivity of 53.2 ± 0.7 mM^{-1} s^{-1}, which represents the highest value so far reported for a Gd(III) complex. The temperature dependence of the relaxivity for the paramagnetic adducts with HSA indicates long exchange lifetimes for the water molecules dipolarly interacting with the paramagnetic centre. This is likely to be related to the formation, upon hydrophobic interaction of the complexes with HSA, of a clathrate-like, second-coordination-sphere arrangement of water molecules. Besides affecting the dissociative pathway of the coordinated water molecule, this water arrangement may itself significantly contribute to enhancement of the bulk solvent relaxation rate.

S. Aime (✉) · M. Botta · M. Fasano · S. Geninatti Crich
E. Terreno
Dipartimento di Chimica Inorganica, Chimica Fisica e Chimica dei Materiali, Università di Torino, via Pietro Giuria 7, I-10125 Turin, Italy
Tel +39-11-6707520; fax +39-11-6707524;
e-mail aime@silver.ch.unito.it

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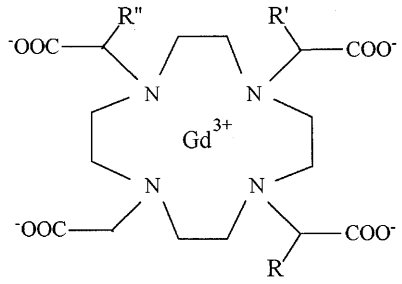
Introduction

Magnetic resonance imaging (MRI), one of the most powerful tools in modern clinical diagnosis, is based on the topological representation of NMR parameters such as proton density and transverse and longitudinal relaxation times. Differences in these parameters allow impressive anatomical discrimination to be made, and make it possible to distinguish pathological from healthy tissues. The potential of MRI is further strengthened by the use of suitable contrast agents (CA), which are compounds able to alter markedly the magnetic properties of the region where they are distributed [1]. Among them, the paramagnetic Gd(III) complexes are under intense scrutiny because of their ability to enhance the proton relaxation rates of solvent water molecules. In order to be usable as a CA for MRI, two basic requirements have to be met: (1) the complex must have at least one coordinated water molecule in fast exchange (on the NMR time scale) with the bulk of the solvent, and (2) the Gd(III) ion must be tightly chelated to avoid the release of the metal ion and the ligand, both potentially harmful. On this basis, GdDOTA (DOTA = 1,4,7,10-tetraazacyclododecane-*N*, *N′*, *N″*, *N‴*-tetraacetic acid) has been shown to be a good CA, as it couples remarkably high thermodynamic and kinetic stabilities with the occurrence of one exchangeable water molecule in its inner coordination sphere. At the magnetic fields usually employed in MRI (0.5–1.5 T), the relaxivity of this complex, namely the solvent proton relaxation rate of a 1 mM solution of the Gd(III) chelate, is essentially determined by the molecular reorientational time (τ_R) of the complex. It was early suggested that an increase in this time, keeping the other parameters involved in the relaxation process unchanged, would cause a large increase in the solvent

proton relaxation rates [2]. This aim has been pursued by linking Gd(III) complexes to large-sized biomolecules such as albumin [3], polylysine [4], polyaspartate [5], and dextran [6] or polymeric species such as dendrimers [7]. However, there is some concern about the toxicity and the metabolic fate of these species [8]. Furthermore, the observed relaxation enhancement was much smaller than that expected on the basis of the paramagnetic relaxation theory, increasing to a value of 19.3 mM^{-1} s^{-1} in the most favourable case of GdDTPA covalently attached to human serum albumin (HSA) through an amide bond [9].

For these reasons, we decided to approach the target of high relaxivities by exploring the route of increasing τ_R through the formation of macromolecular adducts based on the non-covalent interaction between HSA and suitably functionalized DOTA-like Gd(III) complexes. Moreover, the non-covalent interaction with the serum proteins promotes an increased residence time of the contrast agent in the vascular system, and this represents a desirable property for angiographic applications of the MRI techniques. To this end, we have considered four related derivatives of DOTA, containing one, two (*cis*– and *trans*–isomers), and three benzyloxymethyl substituents (Scheme 1), whose relaxometric properties have been previously investigated [10]. The same complexes have already been found to show a significant increase in their relaxivities upon the formation of inclusion complexes with β-cyclodextrin [11] and upon interaction with cationic CTA^+Br^- micelles (CTA = hexadecyltrimethylammonium) [12]. This study was performed using the well-established proton relaxation enhancement (PRE) method that allows both the binding parameters and the relaxivity enhancement ($_b$) of the [Gd(III) complex]-HSA adduct to be determined [13–15]. The introduction of benzyloxymethylic substituent(s) on the surface of the Gd-DOTA complex is expected to give rise to a number of diastereomeric structures in addition to the structural isomers already found in the parent complex [16]. In principle, each isomer is expected to display a different interaction binding to a chiral molecule such as a protein. It follows that our results necessarily represent the average behaviour of the range of isomeric species potentially present for each complex.



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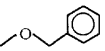
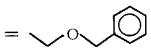
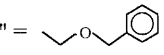
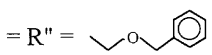
R =  R' = R" = H — GdDOTA(BOM)

R = R' =  R" = H — *cis*-GdDOTA(BOM)$_2$

R = R" =  R' = H — *trans*-GdDOTA(BOM)$_2$

R = R' = R" =  — GdDOTA(BOM)$_3$

Materials and methods

Experimental procedures

HSA (crystallized and lyophilized) was purchased from Sigma (St. Louis, Mo., USA) and was used without any further purification. The molecular weight was assumed to be 69 kDa [17]. (±)-Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] and (S)-(+)-ibuprofen [(S)-(+)-α-methyl-4(2-methylpropyl)phenylacetic acid] were purchased from Aldrich (Milwaukee, Wis., USA). All other chemicals were purchased from Sigma. The synthesis of the ligands and of the Gd(III) complexes was carried out by following the procedure described in [10]. The 50 mM phosphate buffer was prepared by dissolving 1.78 g of $K_2HPO_4 \times 3H_2O$ and 6.64 g of $Na_2H_2PO_4 \times 3H_2O$ in 1 l water. The resulting pH was brought to a value of 7.4 by adding small aliquots of a concentrated solution of NaOH.

The concentration of the aqueous solutions of the Gd(III) complexes was obtained from the known millimolar relaxivity of the paramagnetic complex (R_{1p}^F) [10] by measuring their longitudinal water proton relaxation rates (R_{1obs}) at 20 MHz and 25 °C:

$$R_{1obs} = R_{1p}^F[GdL] + 0.38 \quad (1)$$

where 0.38 s^{-1} is the relaxation rate of pure water at this temperature and magnetic field strength. Longitudinal water proton relaxation rates were measured by the usual (180°-τ-90°) inversion recovery pulse sequence method on a Stelar spin-master spectrometer [Stelar, Mede (PV), Italy] operating at a magnetic field strength of 0.47 T, corresponding to the proton Larmor frequency of 20 MHz. Magnetization values were obtained by averaging the first 128 data points of the free induction decay after four scans. A phase cycle (+*x*, –*x*, –*x*, +x) was applied on the 90° observation pulse to cut off the *y*–scale receiver offset. A reproducibility check (20 measurements) gave an SD of 0.4% in the experimentally determined R_{1obs} values. The built-in temperature controller was calibrated with an external thermometer (±0.1 °C); the samples were allowed to equilibrate in the NMR probe for about 10 min before each measurement. The volume of the solutions employed was 0.06 ml.

Theoretical treatment

The interaction of Gd(III) complexes with HSA has been investigated by measuring the proton relaxation rate of their aqueous solutions. In principle, the longitudinal relaxation rate of the solvent protons (R_{1obs}) of an aqueous solution of a paramagnetic Gd(III) complex is given by the sum of three contributions:

$$R_{1obs} = R_{1p}^{is} + R_{1p}^{os} + R_{1d} \quad (2)$$

where R_{1p}^{is} represents the paramagnetic contribution from the water molecule(s) in the inner coordination sphere of the metal centre, R_{1p}^{os} is the contribution from the water molecules which dif-

fuse near the surface of the paramagnetic complex, and R_{1d} corresponds to the diamagnetic contribution evaluated from an equimolar solution of the corresponding diamagnetic analog (La, Lu and Y complexes).

According to the established theory [18], the inner-sphere contribution is given by:

$$R_{1p}^{is} = \frac{q\,[GdL]}{55.56\,(T_{1M} + \tau_M)} \tag{3}$$

$$T_{1m}^{-1} \propto \frac{D^{is}}{r^6}\, f(\tau_c, \omega_i, \omega_s) \tag{4}$$

$$\tau_C^{-1} = \tau_S^{-1} + \tau_R^{-1} + \tau_M^{-1} \tag{5}$$

where q is the number of metal-coordinated water molecules in the inner coordination sphere, T_{1M} is their longitudinal proton relaxation time, τ_M is their mean residence lifetime, r is the water proton-Gd distance, τ_C is the overall correlation time for the dipolar interaction, τ_R is the reorientational correlation time of the H-Gd position vector, τ_S is the relaxation time of the unpaired electrons of the Gd(III) ion, D^{is} represents the strength of the dipolar interaction, [GdL] is the Gd(III) complex concentration and ω_S and ω_I are the electron and proton Larmor frequencies, respectively. According to Eq. 5, the shortest among τ_R, τ_S and τ_M represents the actual correlation time τ_C, which determines T_{1M} in Eq. 4.

Although for small-sized complexes the outer-sphere contribution R_{1p}^{os} may account for up to 50% of the overall relaxivity, it is not straightforward to foresee to what extent it contributes when the complex is fully bound to a protein. In fact, the simple hard-sphere translational diffusion model which well describes the behaviour of small metal chelates probably plays a marginal role in the case of a macromolecular system. Indeed, in these adducts, water molecules in the hydration shell and mobile protons on the surface of the protein in the proximity of the site of interaction of the paramagnetic complex may significantly contribute to the overall relaxation rate. Since the residence lifetimes of these protons in any given position with respect to the magnetic moment of the Gd(III) ions are expected to be longer than those typical of diffusion processes, this contribution is better defined as a second-sphere term. Thus, the observed relaxation enhancement is actually the result of a cooperative action from inner, second, and outer-sphere contributions. Quantitatively, this enhancement depends upon both the molar fraction of the complex bound to the protein (χ_b) and the relaxivity of the paramagnetic adduct formed by the interaction between HSA and the Gd(III) complex. This increase is expressed by the enhancement factor ε^*:

$$\varepsilon^* = \frac{R_{1p}^*}{R_{1p}} = \frac{R_{1obs}^* - R_{1d}^*}{R_{1obs} - R_{1d}} \tag{6}$$

The asterisk in Eq. 6 indicates the presence in solution of the added macromolecule. The enhancement factor may assume values between 1 (no interaction; $\chi_b = 0$) and ε_b (all GdL bound to HSA; $\chi_b = 1$).

The determination of the binding parameters K_A (association constant) and n (number of independent sites characterized by a given K_A value) for the equilibrium:

$$GdL + HSA \overset{K_A}{\rightleftharpoons} GdL-HSA$$

is possible through the following equations:

$$k_A = \frac{[GdL-HSA)}{[GdL]_F\,[n\,HSA]_F} = \frac{[GdL{\cdot}HSA]}{([GdL]_T - [GdL-HSA])\,([n\,HSA]_T - [GdL-HSA])} \tag{7}$$

$$\varepsilon^* = \frac{[GdL-HSA]}{[GdL]_T}\,\varepsilon_b + \frac{[GdL]_T - [GdL-HSA]}{[GdL]_T} \tag{8}$$

where $[n\,HSA]$ represents the sites concentration, while the T and F subscripts refer to "total" and "free" species, respectively.

Of course, Eq. 8 is valid only if the longitudinal water proton relaxation rate for the free Gd complex is not affected by the presence of the protein in solution. For this reason, the HSA concentration must be lower than the phenomenological limiting value for which the changes in the local microviscosity (which are directly reflected in changes of τ_R and therefore of R_{1p}^{is}) can no longer be neglected. Koenig et al. [19] showed that this limit for a globular protein like haemoglobin is about 3.5 mM, and we think that this limit may be considered reasonable for the serum protein as well. By combining Eqs. 7 and 8 we obtain Eq. 9, which allows the non-linear fitting of the experimental data:

$$\varepsilon^* = (\varepsilon_b - 1)\,\frac{(K_A[GdL]_T + K_A[n\,HSA]_T + 1) - \sqrt{(K_A[GdL]_T + K_A[n\,HSA]_T + 1)^2 - 4K_A^2[n\,HSA]\,[GdL]_T}}{2K_A[GdL]_T} + 1 \tag{9}$$

The experimental procedure consists in the determination of the enhancement factor ε^* through two distinct titrations: E and M. The former reports the change of ε^* as the $[HSA]_T/[GdL]_T$ ratio increases, keeping $[GdL]_T$ constant. The fitting of these experimental data allows the evaluation of ε_b and of the product $K_A \times n$. In the M titration, in contrast, the behaviour of ε^* is measured in solutions containing a fixed protein concentration and variable amounts of GdL complex. The data from this titration are often more conveniently analysed in the form of a Scatchard plot according to Eq. 10 [20].

$$\frac{r}{[GdL]_F} = nK_A - rK_A \tag{10}$$

where r represents the molar binding ratio, i.e. $[GdL\text{-}HSA]/[HSA]_T$. This ratio may easily be calculated, once ε_b is known from the E titration, by using Eq. 8. In the case of a single class of binding sites, a plot of $r/[GdL]_F$ versus r gives a straight line whose x–axis intercept is equal to n, while the slope corresponds to K_A. By assuming that the binding of successive molecules does not alter the K_A value of the bound complex, the curvature of a Scatchard plot implies that there is more than one class of binding sites, each one characterized by its own values of n, K_A and ε_b.

Results and discussion

Evaluation of the diamagnetic contribution R_{1d}^*

In Fig. 1 we report the behaviour of the longitudinal relaxation rates of water protons on increasing the concentration of HSA. A good linear relationship between R_{1d}^* and [HSA] is observed up to concentrations of macromolecule of about 4 mM. The increase of R_{1d}^* with protein concentration is a well-known phenomenon, and it was explained early on in terms of an overall increase in the reorientational correlation time following the exchange of water molecules in te protein hydration shell with the "bulk" solvent [19]. More recently, it has been recognized that an important role has to be ascribed to the exchangeable protons on the surface of the protein [21]. At concentrations >3.5 mM, the likely formation of protein aggregates results in a further decrease in the macromolecular tumbling rate [22]. On this basis, we decided to carry out our study by using

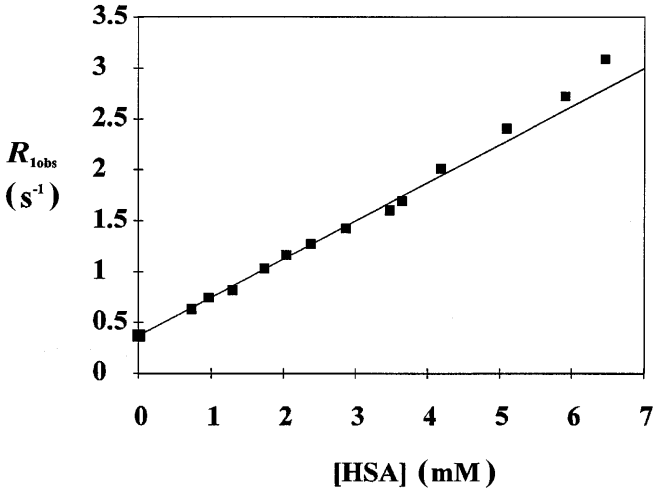


Fig. 1 Effect of the human serum albumin (HSA) concentration on the longitudinal water proton relaxation rate, measured at 20 MHz, 25 °C, pH 7.4, in 50 mM phosphate buffer

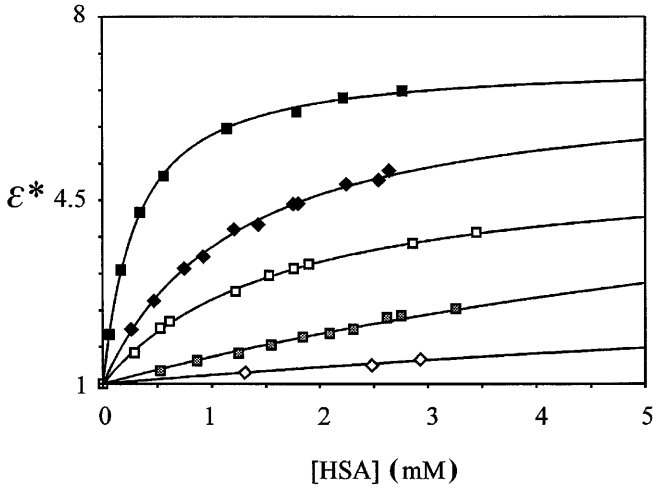


Fig. 2 E titration of a 0.1 mM solution of [Gd-DOTA]$^-$ (◇), [Gd-DOTA(BOM)]$^-$ (▨), Gd-*cis*–DOTA(BOM)$_2$]$^-$ (□), [Gd-*trans*–DOTA(BOM)$_2$]$^-$ (◆), [Gd-DOTA(BOM)$_3$]$^-$ (■), with HSA at 20 MHz, 25 °C, 50 mM phosphate buffer, and pH 7.4. The *solid curves* represent the best fit to Eq. 6

HSA concentrations not higher than 3.5 mM. On the other hand, the albumin concentration in human plasma is close to 0.6 mM.

Determination of K_A and n

In Fig. 2 we report the results of the E titration of the Gd(III) complexes of DOTA and DOTA(BOM), *cis*–DOTA(BOM)$_2$, *trans*–DOTA(BOM)$_2$ and DOTA(BOM)$_3$ ligands. As expected, GdDOTA shows a very small increase in the water proton relaxation rate, which reflects its very weak and aspecific interaction with HSA. All four benzyloxymethyl-substituted DOTA complexes showed an increase in the longitudinal water proton relaxation rate upon addition of HSA. GdDOTA(BOM)$_3$ complex displayed the strongest interaction, as qualitatively inferred by the observation that the relaxation enhancement rises steeply to the asymptotic $_b$ value. The affinity for HSA in this series of related complexes appears to be dependent on the number of pendant hydrophobic substituents on the surface of the ligand. Therefore GdDOTA(BOM) displayed the weakest interaction, whereas *cis*–GdDOTA(BOM)$_2$, and *trans*–GdDOTA(BOM)$_2$ showed intermediate behaviour (see Table 1). For the weakly interacting Gd-DOTA(BOM) complex, the fitting procedure only allows us to evaluate an upper limit for the binding constant, i.e. $K_A < 1 \times 10^2\ M^{-1}$. The millimolar relaxivities of GdL-HSA adducts (R_{1p}^B) may easily be obtained by multiplying $_b$ by R_{1p}^F. These values are generally remarkably high and, in particular in the case of GdDOTA(BOM)$_3$–HSA adduct, the obtained value of 53.2 ± 0.7 mM s^{-1} represents the highest relaxivity so far reported for a Gd(III) complex.

For the application in MRI, it may be useful to compare the observed relaxation rates of the three complexes at the physiological concentration of HSA (0.6 mM) (Table 1). Clearly, the higher binding affinity of GdDOTA(BOM)$_3$ makes it possible to obtain quite a high relaxation rate at this HSA concentration also, as its bound form to the protein raises the value by 65.6%. For the three complexes *cis*–GdDOTA(BOM)$_2$, *trans*–GdDOTA(BOM)$_2$ and GdDOTA(BOM)$_3$, whose E titrations indicated a strong interaction with HSA, we proceeded to the M titration in order to assess both n and K_A. As reported in Fig. 3, the titration curves obtained for the three complexes appear consistent with the occurrence of a single class of binding sites with $n = 2$. We checked this result by fitting the M titration data by releasing the equivalence condition in

Table 1 Binding parameters and relaxivities measured with human serum albumin (*HSA*) at 25 °C, 20 MHz and pH 7.4

Gd(III) complex	K_A (M^{-1})	n	$_b$	R_{1p}^F ($mM^{-1}\,s^{-1}$)	R_{1p}^B ($mM^{-1}\,s^{-1}$)	R_1 (s^{-1})[b]
GdDOTA(BOM)	$<1\cdot10^2$	[a]	[a]	5.4 ± 0.1	[a]	1.3
cis-GdDOTA(BOM)$_2$	$3.2 \pm 0.4 \cdot 10^2$	2	5.2 ± 0.1	6.8 ± 0.1	35.7 ± 0.9	2.0
trans-GdDOTA(BOM)$_2$	$3.6 \pm 0.4 \cdot 10^2$	2	6.8 ± 0.1	6.5 ± 0.1	44.2 ± 1.3	2.3
GdDOTA(BOM)$_3$	$1.7 \pm 0.1 \cdot 10^3$	2	7.1 ± 0.1	7.5 ± 0.1	53.2 ± 1.5	4.3

[a] Not determined since only an upper limit for K_A can be obtained
[b] These values are referred to solutions containing 0.1 mM of paramagnetic complex at physiological concentration (0.6 mM) of HSA

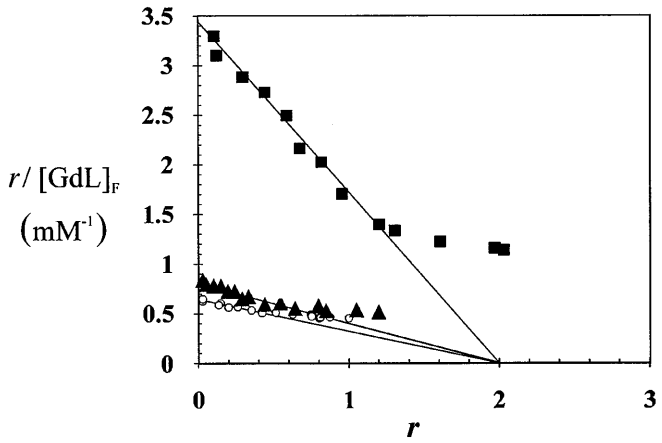


Fig. 3 Scatchard plots for the binding to HSA of [Gd-DOTA-$(BOM)_3]^-$ (■), [Gd-*cis*–$DOTA(BOM)_2]^-$ (○) and [Gd-*trans*–$DOTA(BOM)_2]^-$ (▲) as measured at 20 MHz, 25 °C, pH 7.4, and [HSA] 0.6 mM. The *straight lines* through the data points represent the best fit to Eq. 7 using the data points with [GdL]/[HSA] < 1 only

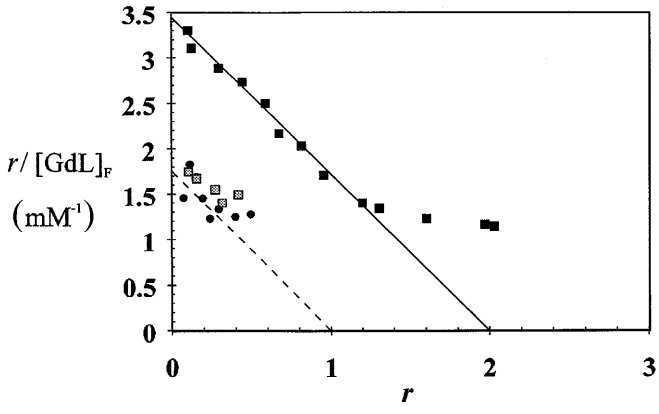


Fig. 4 Scatchard plots for the binding to HSA of [Gd-DOTA-$(BOM)_3]^-$ alone (■) or in combination with displacer drugs [ibuprofen 0.6 mM (▨); warfarin 0.6 mM (●)] measured at 20 MHz, 25 °C, 50 mM phosphate buffer, pH 7.4, and [HSA] = 0.6 mM. The *dotted straight line* was calculated assuming $n = 1$ and $K_A = 1.7 \times 10^3\ M^{-1}$

order to provide an estimate of the largest possible difference between the two binding constants. In the case of $GdDOTA(BOM)_3$, it was found that the fit becomes unacceptably bad when the chosen values are outside the range $1.4–2.5 \times 10^3\ M^{-1}$ for K_A and 6.5–7.6 for $_b$ respectively. Thus, two nearly equivalent binding sites are available on HSA for these complexes in spite of the irregular structure of the protein. Furthermore, the slope change in the binding curves at higher complex/HSA ratios indicates the presence on the protein surface of other weaker, and probably non-specific, binding sites [23–25].

The binding parameters calculated for the two strong binding sites to HSA of the three complexes investigated are reported in Table 1. These data outline the qualitative conclusions drawn from the E titrations reported in Fig. 1 and stress the important role of the number of hydrophobic substituents in determining the interaction strength. The binding strength of $GdDOTA(BOM)_3$ complex to HSA is of the same order as that reported for endogeneous substances like testosterone and aldosterone [26, 27].

Investigation of the binding sites

It is well known that HSA can interact with many substrates displaying quite different structural features. This behaviour has been accounted for in terms of the absence on the macromolecule of specific binding sites characterized by a high structural affinity with the substrate molecules; rather there are extended regions able to accommodate structurally different species [28]. From this point of view, the binding of most of the exogenous substrates occurs primarily in two subdomains (IIA and IIIA) of HSA [29], which are characterized by the occurrence of a hydrophobic pocket surrounded by a positively charged external surface and accessible to the solvent molecules. These structural features explain the affinity of albumin in binding small anionic-hydrophobic compounds, and we would then expect that the interacting sites of our Gd(III) complexes should lie in these subdomains. To check this hypothesis, competition assays have been carried out between $GdDOTA(BOM)_3$ complex and two drugs, warfarin and ibuprofen, which are known to be specific probes for subdomains IIA and IIIA respectively [30, 31]. These experiments have been performed by measuring the relaxation enhancement factor * upon addition of successive aliquots of $GdDOTA(BOM)_3$ to solutions containing HSA and the specific probe molecule (warfarin or ibuprofen) in the stoichiometric ratio 1:1. Since both the competitor probes bind HSA with K_A values of about two orders of magnitude higher than that of the $GdDOTA(BOM)_3$ complex [32, 33], their sites should no longer be available to the Gd(III) complex. In fact, as shown in Fig. 4, in both experiments the Scatchard plot of the M titrations indicates a single binding site with K_A values similar to those measured in the absence of warfarin and ibuprofen (dotted line in the figure).

Complete displacement of $GdDOTA(BOM)_3$ from the protein has been observed when equimolar amounts of warfarin and ibuprofen (0.6 mM) are simultaneously added to a solution containing the complex (0.1 mM) and the protein (0.6 mM). In fact the observed R_{1p} value decreased from 4.3 (* = 5) to 1.9 s^{-1} (* = 1.7); the residual enhancement is due to the contribution arising from the aspecific interaction of $GdDOTA(BOM)_3$ at the weak binding sites on the surface of the protein. Thus, we conclude that both sites of interaction of $GdDOTA(BOM)_3$ with HSA are located, analogously to warfarin and ibuprofen, in subdomains IIA and IIIA.

Thermodynamic parameters of the interaction with HSA

In order to obtain more insight into the driving forces responsible for the interaction of these Gd(III) complexes with HSA, we measured the K_A values for *cis*–GdDOTA(BOM)$_2$, *trans*–GdDOTA(BOM)$_2$, and GdDOTA(BOM)$_3$ in the 283–312 K temperature range. The results obtained, analysed in terms of the van't Hoff equation were used for the evaluation of the thermodynamic parameters (ΔH° and ΔS°) characterizing the interaction (Fig. 5, Table 2). A positive value of ΔS° is typical of a hydrophobic interaction, and it has sometimes been explained as the result of the so-called iceberg effect [34]. In line with this suggestion, the hydrophobic binding of the Gd(III) complexes to HSA would cause the disruption of the ordered arrangement of water molecules around the protein and the complex. The increase in entropy reflects the decreased order of the water arrangement around the protein-Gd(III) complex adduct. In the case of *trans*–GdDOTA(BOM)$_2$ and GdDOTA(BOM)$_3$ complexes, this entropic contribution largely dominates the ΔG° term. The small negative enthalpies primarily reflect the electrostatic interactions between the negatively charged complexes and the positively charged groups on the macromolecule.

The significant differences in ΔH° and ΔS° between the *cis*- and *trans*- GdDOTA(BOM)$_2$ isomers clearly reflect substantial changes in their binding mode to HSA, but it is not possible on the basis of the information available to attempt any further speculation on the structural differences between the two adducts.

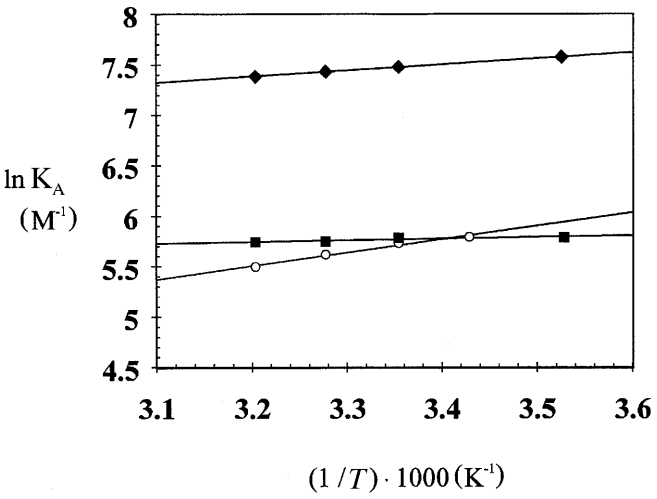


Fig. 5 Van't Hoff plots for the binding to HSA of [Gd-DOTA-(BOM)$_3$]$^-$ (◆), [Gd-*cis*–DOTA(BOM)$_2$]$^-$ (○), and [Gd-*trans*–DOTA(BOM)$_2$]$^-$ (■). The *straight lines* represent the best fit to the van't Hoff equation

Effect of temperature on the observed relaxation rates

In the context of the potential application of these Gd(III) complexes as CA for MRI, the PRE method has the obvious advantage (with respect to the other available approaches to the study of the interaction with macromolecules) of being based on the measurement of the relaxation enhancement itself, which is the most demanding requirement to be considered for this application. Thus, in this section we attempt to provide an insight into the various contributions to the factors determining the observed relaxation enhancement and also into their temperature dependence.

At any temperature, the observed relaxation rate is given by the sum of three contributions:

$$R_{1obs} = R_{1p}^{F}[GdL]_F + R_{1p}^{B}[GdL-HSA] + R_{id} \quad (11)$$

A knowledge of K_A, R_{1p}^{F} and R_{1d}^{*} at the various temperatures allows us to evaluate the individual contributions of the three terms of Eq. 11 to the observed relaxation rates. In Fig. 6 we report the resolution of the temperature dependence of R_{1obs} into the profiles of its components for a solution containing GdDOTA-(BOM)$_3$ (0.1 mM) and HSA (2.9 mM). In this solution, the observed relaxation rate is largely dominated by the paramagnetic contribution arising from the GdDOTA-(BOM)$_3$–HSA adduct. Very interesting was the observation of a bell-shaped profile for the last contribution, with a maximum value near 40 °C. Note, in contrast, that the profiles of the contributions arising from the free Gd(III) complex and from the diamagnetic HSA have the expected exponential decrease upon temperature increase as a result of the shortening of the molecular reorientational time τ_R.

The same procedure has been applied to HSA solutions containing *cis*–GdDOTA(BOM)$_2$ and *trans*–GdDOTA(BOM)$_2$. In Fig. 7 we report the temperature dependence of the millimolar relaxivities of their GdL-HSA adducts. The profiles for *trans*–GdDOTA-(BOM)$_2$–HSA and GdDOTA(BOM)$_3$–HSA are rather similar and recall the close similarity of their values of ΔH° and ΔS°, whereas the profile of the adduct with *cis*–GdDOTA(BOM)$_2$ shows a steeper increase of R_{1p}^{B} with temperature. It is likely that the value reached at 55 °C represents a relaxivity maximum, although this cannot be proved because of the incipient denaturation of the protein at higher temperatures.

Table 2 Thermodynamic parameters as measured at pH 7.4

Gd(III) complex	ΔH° (kcal/mol)	ΔS° (cal mol^{-1} K^{-1})	ΔG^{298} (kcal/mol)
cis-GdDOTA(BOM)$_2$	−2.64 ± 0.01	2.5 ± 2.1	−3.4 ± 0.6
trans-GdDOTA(BOM)$_2$	−0.31 ± 0.01	10.4 ± 1.9	−3.4 ± 0.6
GdDOTA(BOM)$_3$	−1.18 ± 0.01	10.9 ± 1.4	−4.4 ± 0.4

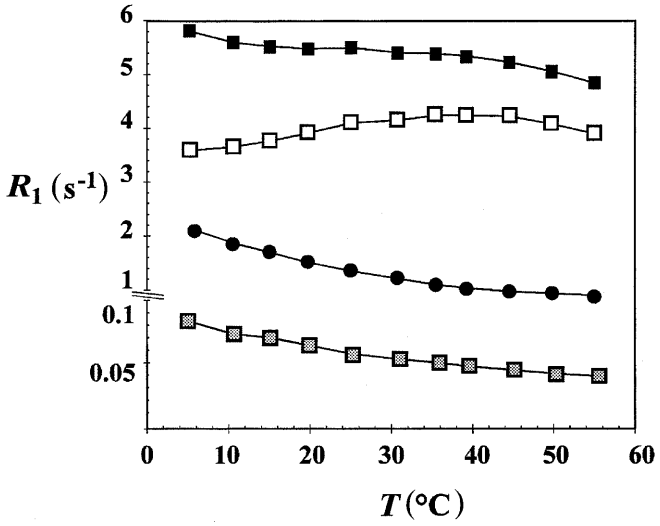


Fig. 6 Temperature dependence of the longitudinal water proton relaxation rate for a 0.1 mM solution of $[Gd\text{-}DOTA(BOM)_3]^-$ in the presence of HSA 2.9 mM (20 MHz; 50 mM phosphate buffer; pH 7.4): observed values (■), diamagnetic (●) and paramagnetic contribution for free (▨) and bound (□) complex

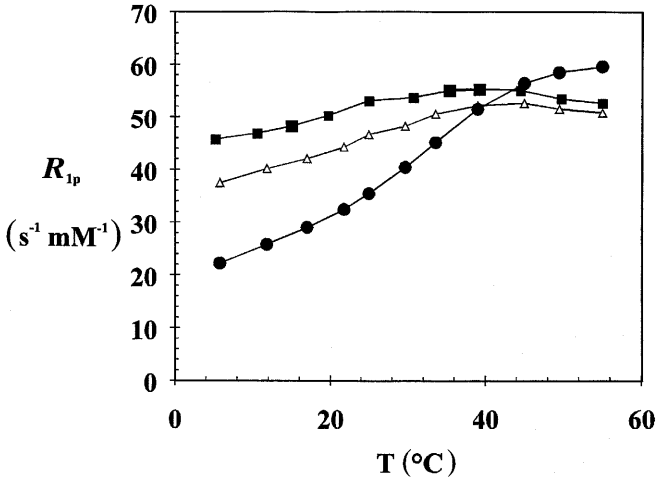


Fig. 7 Temperature dependence of the relaxivity of HSA adducts of $[Gd\text{-}DOTA(BOM)_3]^-$ (■), [Gd-*cis*–$DOTA(BOM)_2]^-$ (●), and [Gd-*trans*–$DOTA(BOM)_2]^-$ (△), measured at 20 MHz, 25 °C, 50 mM phosphate buffer, and pH 7.4

In terms of the theory described above, the observed behaviour appears indicative of a τ_M dependence of R_{1p}^{is} as defined by Eq. 3. At low temperatures, τ_M is longer that T_{1M} and dominates R_{1p}. On increasing the temperature, τ_M becomes shorter and R_{1p} increases until the condition $\tau_M \geq T_{1M}$ holds. Thus, the relaxation enhancement promoted by the increase of τ_R upon the formation of the large paramagnetic adduct with HSA appears to be quenched by the occurrence of a long exchange lifetime of the coordinated water molecule. In simple aqueous solutions, the water exchange lifetime has been found to be a limiting factor with respect to the relaxivity of Gd(III) complexes only in the case of neutral bisamide DTPA-like Gd(III) complexes [35–39]. However, Merbach and coworkers [35–37] have also shown that the occurrence of relatively long τ_M values is a common feature in Ln(III) complexes containing one coordinated water only, and this has been related to the dissociative mechanism operating in this class of complexes.

The data in Fig. 7 suggest that the adduct of HSA with *cis*–$GdDOTA(BOM)_2$ has a τ_M value longer than that with *trans*–$GdDOTA(BOM)_2$. On the basis of the smaller $\Delta S°$ value shown by the former adduct, one may speculate that the longer τ_M value may be associated with a more ordered arrangement of the water molecules at the binding site. Recent studies have shown that the exchange rate in the clathrate of water molecules upon formation of a hydrophobic interaction may drastically decrease [40]. Since this clathrate of water molecules embeds the outer region of the complex, it is likely that it has a relevant role in slowing down the dissociation rate of the inner sphere water molecules. Thus, the long τ_M value associated with the hydrophobic interaction of a complex with HSA may be the result of the ordered arrangement of water molecules in the region of contact between the complex and the macromolecule.

The formation of this clathrate-like, second-coordination sphere arrangement of water molecules may also deeply affect the overall solvent relaxation rate, as their residence lifetimes in the proximity of the paramagnetic centre are now much longer than those characterizing the diffusion processes in the typical outer-sphere model. This is what probably occurs with tetraphosphinate DOTA-like Gd(III) complexes that, although having $q = 0$, show a marked relaxation enhancement upon interaction with albumin [41].

Also highly pertinent to this discussion are the results obtained by Kushnir and Navon [42] in the study of water relaxation rates in solutions of paramagnetic metalloenzymes. They found that a large contribution to the overall relaxation enhancement arises from water molecules in the second-coordination sphere. On this basis, we may look at the Gd(III) complex/HSA adducts in terms of an "artificial" metallo-protein where both the paramagnetic metal and the hydrophobic cavity are provided by the functionalized complex. The number of hydrophobic substituents, besides determining the strength of the interaction, affects the dynamics of the second-sphere water molecules. Of course, a better understanding of these features may be very important in the design of novel paramagnetic Gd(III) complexes of enhanced efficacy and selectivity with respect to interaction with biomolecules.

Concluding remarks

The results here reported show that it is possible to achieve a high relaxivity enhancement by exploiting the non-covalent interaction between suitably functional-

ized Gd(III) complexes and HSA. On the one hand, this will allow a marked reduction in the administered doses of contrast agent, and, on the other hand, it will make it possible to design novel angiographic experiments for which an increased residence time in the circulating blood is required. In this regard, we have shown that it is possible to modulate the extent of the interaction with the macromolecule by varying the number of hydrophobic substituents on the surface of the ligand.

The quenching effect on the overall relaxivity enhancement caused by the long exchange lifetime of water molecules in the proximity of the paramagnetic centre could possibly be avoided by the use of complexes with $q = 2$. In fact, it is expected that for these complexes exchange pathways of lower energy for the coordinated water molecules without a significant decrease in their stability will be observed [43, 44].

An interesting insight from the observations reported in this paper is the recognition of the additional contribution to the solvent relaxation rate arising from second-sphere water molecules surrounding the interaction site. This effect may be very important for the further improvement of the relaxation enhancement promoted by the increase in the reorientational correlation time. In summary, we believe that the body of the information gained in this work points the way towards novel "tailored" relaxation agents for MRI applications.

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References

1. Engelstad BL, Wolf GL (1988) Contrast agents. In: Stark DD, Bradley Jr WG (eds) Magnetic resonance imaging. Mosby, St. Louis, pp 161–181
2. Lauffer R.B. (1987) Chem Rev 87:901–927
3. Brasch R C (1991) Magn Reson Med 22:282–287
4. Manabe Y, Longley C, Furmansky P (1986) Biochim Biophys Acta 883:460–467
5. Cavagna F, Luchinat C, Scozzafava A, Xia Z (1994) Magn Reson Med 1:58–60
6. Armitage FE, Richardson DE, Li KCP (1990) Bioconjug Chem 1:365–374
7. Wiener EC, Brechbiel MW Brothers H, Magin RL, Gansow OA, Tomalia DA, Lauterbur PC (1994) Magn Reson Med 1:1–8
8. Sherry AD, Cacheris WP, Kah-Tiong Kuan (1988) Magn Reson Med 8:180–190
9. Lauffer RB, Brady TJ, Brown RD III, Baglin C, Koenig SH (1986) Magn Reson Med 3:541–548
10. Aime S, Botta M, Ermondi G, Fedeli F, Uggeri F (1992) Inorg Chem 31:1100–1103
11. Aime S, Botta M, Panero M, Grandi M, Uggeri F (1991) Magn Reson Chem 29:923–927
12. Aime S, Barbero L, Botta M (1991) Magn Reson Imaging 9:843–847
13. Dwek RA (1973) Nuclear magnetic resonance in biochemistry, application to enzyme systems. Clarendon Press, Oxford, pp 247–284
14. Jenkins BG, Armstrong E, Lauffer RB (1991) Magn Reson Med 17:164–178
15. Mildvan AS, Cohn M (1963) Biochemistry 2:910–919
16. Aime S, Botta M, Ermondi G (1992) Inorg Chem 31:4291–4299
17. Peters Jr T (1986) Adv Protein Chem 37:161–245
18. Bertini I, Luchinat C (1986) NMR of paramagnetic molecules in biological systems. Benjamin/Cummings, Menlo Park
19. Koenig SH, Brown III RD (1990) Prog NMR Spectr 22:487–567
20. Scatchard G (1949) Ann N Y Acad Sci 51:660–672
21. Liepinsh E, Otting G (1996) Magn Reson Med 35:30–42
22. Sadler PJ, Tucker A (1992) Eur J Biochem 205:631–643
23. Zini R, D'Athis P, Barre J, Tillment JP (1979) Biochem Pharmacol 28:2661–2665
24. Hsia JC, Song SEn, Tan CT, Tinker DO (1982) J Biol Chem 257:1724–1729
25. Larsen SK, Jenkins BG, Memon NG, Lauffer RB (1990) Inorg Chem.29:1147–1152
26. Kragh-Hansen U (1981) Pharmacol Rev 33:17–53
27. Chanut E, Zini R, Trouvin JH, Riant P, Tillement JP, Jacquot C (1992) Biochem Pharmacol 44:2082–2085
28. Kragh-Hansen U (1990) Dan Med Bull 37:57–84
29. Carter DC, Ho JX (1994) Adv Protein Chem 45:153–203
30. Min He X, Carter DC (1992) Nature 358:209–215
31. Oida T (1986) J Biochem 100:99–103
32. Kasai-Morita S, Horie T, Awazu S (1987) Biochem Biophys Acta 915:277–283
33. Rahman MH, Maruyama T, Okada T, Imai T, Otagiri M (1993) Biochem Pharmacol 46:1733–1740
34. Tanaka M, Asahi Y, Masuda S, Ota T (1989) Chem Pharmacol Bull 37:3177–3180
35. Micksei K, Helm L, Brucher E, Merbach E (1993) Inorg Chem 32:3844–3850
36. Gonzalez G, Powell DH, Tissieres V, Merbach AE (1994) J Phys Chem 98:53–60
37. Powell DH, Favre M, Graeppi N, Ni Dhubhghaill OM, Pubanz D, Merbach AE (1995) J Alloy Comp 225:246–252
38. Aime S, Botta M, Fasano M, Paoletti S, Anelli PL, Uggeri F, Virtuani M (1994) Inorg Chem 33:4707–4711
39. Aime S, Fasano M, Paoletti S, Terreno E (1995) Gazz Chim Ital 125:125–131
40. Spyracopoulos L, O'Neil JDJ (1994) J Am Chem Soc 116:1395–1402
41. Aime S, Batsanov AS, Botta M, Howard JAK, Parker D, Senanayake K, Williams G (1994) Inorg Chem 33:4696–4706
42. Kushnir T, Navon G (1984) J Magn Reson 56:373–384
43. Graeppi N, Powell DH, Laurenczy G, Zékány L, Merbach AE (1995) Inorg Chim Acta 235:311–326
44. Aime S, Botta M, Geninatti Crich S, Giovenzana GB, Jommi G, Pagliarin R, Sisti M (1995) Chem Commun 1885–1886