Maximizing the relaxivity of HSA-bound gadolinium complexes by simultaneous optimization of rotation and water exchange†

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Two new GdEGTA (EGTA = ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) derivatives incorporating aromatic moieties into the oxoethylenic bridge have been prepared and characterised, their conjugates to HSA investigated and an unprecedented high relaxivity, close to that predicted by theory, interpreted in terms of the combined effect of restricted local rotation and fast rate of water exchange.

The advent and the successive rapid development of MRI into a prominent diagnostic modality have seen a parallel and intense activity in chemical research aimed at the design of effective, specific and safe contrast enhancing agents (CA's).[1,2] These are often required in order to improve the diagnostic content of the tomographic image and shorten its acquisition time, intrinsically long because of both the poor sensitivity of NMR, particularly at the clinically used magnetic field strengths, and the long values of the nuclear magnetic relaxation times, $T_{1,2}$, of the ^{1}H nuclei of tissues. The T_1's for the water protons in tissues being much longer than T_2's, T_1-specific CA's were soon established as the most efficient systems and were the first to be clinically employed. The gadolinium complexes of polyaminocarboxylate ligands are the most common of these agents since they satisfy most of the requirements necessary for safe *in vivo* use.[3] Although the control of parameters such as solubility, stability, inertness, biodistribution and excretion pathways by suitable modifications of the chelate basic structures has seen significant improvements over the years, the efficacy of the Gd^{III} chelates to catalyze the water protons' relaxation times has not increased in a comparable manner.[4] This efficacy depends on a number of parameters that describe the modulation of the dipolar interaction between the metal ion and the proton nuclei of the water molecules belonging to its inner-, second- and outer-spheres of hydration.[2,4,5] Two of the most important are the rate of exchange, $k_{ex} = 1/\tau_M$, of the coordinated water molecule(s) and the rate of reorientation, $1/\tau_R$, of the system. Theory predicts that the gadolinium complexes endowed with optimal values of τ_M and τ_R should have a relaxivity, r_{1p}, between *ca.* 80 and 120 mM^{-1} s^{-1} (per bound water molecule and per Gd, at 20 MHz and 298 K), depending on the electronic relaxation parameters.[1–3] At this frequency the optimal residence lifetime can be set around 30 ns, while most of the complexes based on DTPA and DOTA ligands present τ_M values between *ca.* 70 and 1000 ns.[6] A slow rate of water exchange corresponds to an inefficient transfer of the paramagnetic effect from the Gd^{III} centre to the bulk water and such an effect can severely limit the relaxivity in the case of macromolecular systems. This is clearly exemplified by the nearly linear dependence of the relaxivity of several Gd^{III} complexes non-covalently bound to human serum albumin (HSA) and k_{ex}.[7] As a consequence, the relaxivity of these adducts is generally limited to the range 20 to 50 mM^{-1} s^{-1}, *i.e.* much lower than expected on the basis of the increase in molecular size (slower tumbling rate, longer τ_R).

However, more recently it has become evident that another important factor opposes the attainment of high relaxivities in macromolecular conjugates: the presence of a relatively fast internal rotation superimposed on a global reorientation of the system. The Gd^{III}-based CA's are typically conjugated to proteins, dendrimers, micelles and virus capsids through a targeting group connected to the coordination cage with a linker. The possible internal rotation about the linker introduces a degree of flexibility that results in an effective reorientational correlation time of the Gd^{III}–water–proton vector shorter than that associated with the global rotation of the system.[4,8–11] As a consequence any increase in the molecular size does not translate into a proportional decrease of the tumbling motion and then into a relaxivity enhancement. Whereas a few possible strategies to overcome this problem have been discussed,[4,10,12] a clear and definitive example is still lacking of a macromolecular complex that combines a fast exchange of the inner-sphere water with an effective coupling between the Gd–water vector and the tumbling motion of the whole system.

A suitable and simple model should consist of a complex characterized by small size, compactness, one inner-sphere water molecule with a fast rate of exchange and by the presence of a rigid targeting moiety capable of interacting with HSA. To this purpose we designed two new complexes derived from the basic structure of GdEGTA, since this compound was shown to present an optimal τ_M value of 30 ns (298 K) explained in terms of steric compression at the binding site induced by the oxoethylenic bridge.[13] In the new **L1** and **L2** ligands (Scheme 1) the basic structure of EGTA was modified in the central ethylenic moiety, rigidified by fusion with an aromatic ring. The modified ligands were prepared from 1,2-arenediols. **L1** was obtained from 2,3-naphthalenediol, whereas

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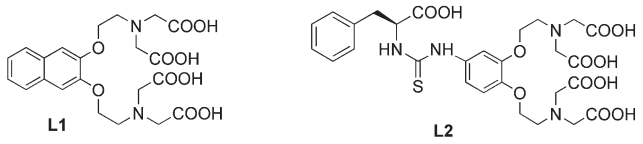

Scheme 1

L2 was prepared from 4-nitrocatechol, in which the nitro group, once reduced, represents a useful site for further functionalization. The naphthalenic backbone or the pendant hydrophobic arm are also useful to exploit conjugation with HSA. Gd**L1** represents our best model and Gd**L2** a closely related complex only differing by the presence of a flexible pendant group. It must be noted that the kinetic and thermodynamic stabilities of these complexes are probably not high enough to permit their safe *in vivo* use and thus Gd**L1** and Gd**L2** should be considered as model compounds for a study intended to be a proof of principle.

The water exchange rate at the Gd centre was assessed by the well established VT ^{17}O NMR procedure,[14] measuring the temperature dependence of R_2 at 2.12 T. For both complexes rather similar exchange rates were obtained ($\tau_M \cong 17 \pm 3$ ns at 298 K) nearly twice as fast as with GdEGTA (Table 1). It is worth noting that, in spite of their similar exchange rates the two new derivatives are endowed with negative values of the activation entropy. This suggests the interesting possibility of a change in the exchange mechanism that requires further investigation by variable pressure ^{17}O NMR. In any case, the presence of the aromatic group in the ligand backbone does not modify the hydration number (q = 1) of the complexes. This is confirmed by the relaxivities of 5.7 and 7.0 mM^{-1} s^{-1} (298 K, pH 7.2, 20 MHz) measured for Gd**L1** and Gd**L2** respectively, typical of monohydrated Gd-complexes and strictly proportional to their molecular weight.[5] Unlike the parent GdEGTA complex, the presence of aromatic moieties allows the new complexes to set up non-covalent interactions with the hydrophobic sites of HSA. The affinity constants, K_A, and relaxivities of the adducts with the protein, r_{1p}^b, were determined, at 20 MHz and 298 K, from the analysis of the data obtained from titration experiments of dilute solutions of the complexes with HSA (Table 2).[7]

Both complexes present similar and weak affinity constants as expected on the basis of the chemical nature of the binding moieties, but they significantly differ in the relaxivities of their adducts, estimated to be 68 ± 2 and 45 ± 3 mM^{-1} s^{-1} for Gd**L1** and Gd**L2** respectively. The different relaxivities can be easily attributed to the expected different degree of local flexibility of the two complexes at the binding site. Whereas an r_{1p}^b value of 45 mM^{-1} s^{-1} is similar to that previously observed in the case of DOTA derivatives featuring benzyloxymethylenic pendant groups, the relaxivity of 68 mM^{-1} s^{-1} is the highest so far reported for a Gd-complex conjugated to a protein. At 310 K the r_{1p}^b value is 65 mM^{-1} s^{-1}, *ca.* 28% higher than for GdMS-325-HSA.[12] The field dependence of the water proton relaxivities was measured for dilute solutions of the complexes (0.11 and 0.10 mM for Gd**L1** and Gd**L2** respectively) in the absence and in the presence of HSA (≈ 1.7 mM) at 298 K in the frequency range 2–70 MHz. These so-called nuclear magnetic relaxation dispersion (NMRD) profiles allow a detailed characterization of the paramagnetic solutes in terms of a large set of structural and dynamic parameters.[15] From the values of K_A, the relaxivities of the free complexes and the diamagnetic contribution of the protein we calculated the r_{1p}^b values at each frequency (Fig. 1). The shape of the profiles of the two adducts are very similar, with a rather narrow peak at *ca.* 30 MHz, typical of slowly tumbling systems. The highest relaxivity values are 78 and 50 mM^{-1} s^{-1} for Gd**L1**–HSA and Gd**L2**–HSA respectively. Clearly, the relaxivity gain in physiological conditions is expected to be sensibly lower due to the relatively weak protein binding. The data were fitted to the Lipari–Szabo model-free approach that takes into account the presence of an internal rotation, characterized by a correlation time τ_{RL}, superimposed on a global motion described in terms of the correlation time τ_{RG}.[16] The degree of correlation between the two motions is described by the parameter S^2 whose value is zero when the two motions are completely independent; in the absence of local fluctuations the complex is immobilized and S^2 = 1. The value of the parameter τ_{RG} was fixed to 40 ns in order to account for the global

Table 1 Selected best-fit parameters obtained from analysis of the ^{17}O NMR data (2.12 T)

Parameter	GdEGTA[a]	Gd**L1**	Gd**L2**
$^{298}k_{ex}/10^7$ s^{-1}	3.1 ± 0.2	5.3 ± 0.3	6.1 ± 0.4
$\Delta H^{\#}$/kJ mol^{-1} [b]	42.7 ± 3.1	17.3 ± 2.9	16.0 ± 2.3
$\Delta S^{\#}$/J mol^{-1} K^{-1} [c]	+42 ± 3	−39 ± 2	−42 ± 3
$(A)/10^6$ rad s^{-1} [d]	−3.2 ± 0.1	−3.6 ± 0.2	−3.5 ± 0.1
$\Delta^2/10^{19}$ s^{-2} [e]	3.4 ± 0.2	4.5 ± 0.1	4.6 ± 0.3
τ_V/ps[f]	24 ± 1	17 ± 2	18 ± 1

[a] Data from ref. 13. [b] Activation enthalpy of the exchange process. [c] Activation entropy of the exchange process. [d] Scalar (hyperfine) coupling constant. [e] Trace of the squared zero field splitting (ZFS) tensor. [f] Correlation time of the modulation of the transient ZFS.

Table 2 Selected parameters obtained from the analysis of the relaxometric titrations (20 MHz; 298 K) and $1/T_1$ NMRD profiles (298 K) of Gd**L1** and Gd**L2** with HSA

Parameter	Gd**L1**	Gd**L2**
K_A/M^{-1}	880 ± 100	940 ± 90
$^{298}r_{1p}/mM^{-1}$ s^{-1} [a]	5.7 ± 0.2	7.0 ± 0.3
$^{298}r_{1p}^b/mM^{-1}$ s^{-1} [b]	68 ± 2	45 ± 3
τ_{RL}/ns	6.0 ± 1.7	1.1 ± 0.2
τ_{RG}/ns[c]	41	41
S^2	0.60 ± 0.13	0.31 ± 0.05

[a] Relaxivity of the free complex (20 MHz). [b] Relaxivity of the HSA-bound complex (20 MHz). [c] Fixed during the fitting.[16]

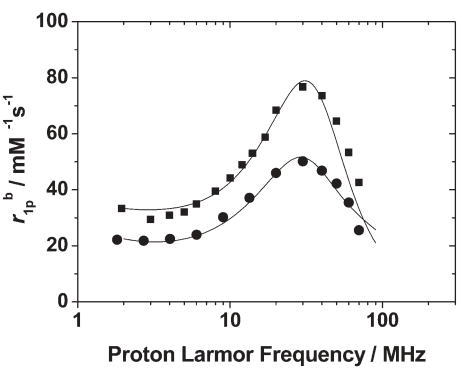

Fig. 1 $1/T_1$ NMRD profiles and fits for Gd**L1**–HSA (squares) and Gd**L2**–HSA (circles) at 298 K.

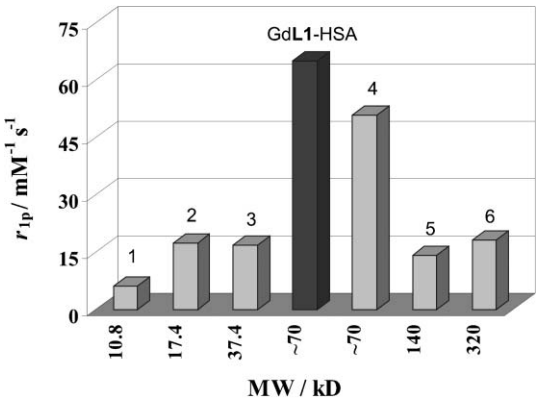

Fig. 2 Plot of the proton relaxivity r_{1p} for selected macromolecular Gd-complexes at 20 MHz and 310 K. Data taken from ref. 1*b*.(1) GdDTPA–PEG I–polylysine (linear synthetic polymer); (2) Gadomer 17 (dendrimer) (3) G_4([NCS]N-bz-GdDO3A)$_{38}$ (dendrimer); (4) MS-325-HSA (non-covalently bound to HSA adduct); (5) Albumin–GdDTPA (covalently bound to HSA macromolecular system); (6) MPEG–PL–GdDTPA (linear synthetic polymer).

reorientation of the protein,[17] whereas τ_{RL} and S^2 were used as variable parameters. The results are given in Table 2 and are in full agreement with the expected higher degree of rigidity of Gd**L1** at the binding site, as indicated by the significantly longer values of τ_{RL} (6.0 *vs.* 1.1 ns) and S^2 (0.60 *vs.* 0.31).

Preliminary molecular modelling studies based on docking of Gd**L1** and Gd**L2** to HSA are in agreement with relaxometric experimental results. Gd**L1** is held fixed inside the entrance of the binding pocket, allowing very small rotational movements. On the other hand, Gd**L2** is more flexible and the interaction with the HSA binding pocket leaves the cage free to rotate outside the binding site, explaining the shorter τ_{RL} for this complex. Thus, the different rotational dynamics arising from the different flexibility of the targeting groups fully accounts for the different observed relaxivities of Gd**L1** and Gd**L2** bound to HSA.

This work shows for the first time that it is possible to achieve relaxivity values very close to those predicted by theory by controlling not only the rate of water exchange but also the rotational dynamics of the system. The relevant result is the remarkable gain in relaxivity observed (per Gd and per coordinated water molecule) for a model complex, unprecedented for macromolecular conjugates of similar or even larger molecular weight (Fig. 2). Finally, these results suggest that a further refinement of the electronic relaxation should allow attainment of relaxivities close to or above 100 mM^{-1} s^{-1}.

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