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Full Paper

A gadolinium triacetic monoamide DOTA derivative with a methanethiosulfonate anchor group. Relaxivity properties and conjugation with albumin and thiolated particles

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ABSTRACT: The gadolinium(III) complex with a new DOTA-based ligand bearing a methanethiosulfonate group (MTS) was synthesized and its relaxivity properties were investigated. MTS-ADO3A is a triacid DOTA derivative with an amide arm substituted by an ethylmethanethiosulfonate function. This ligand was obtained in two steps: tri-*tert*-butyl 2,2′,2″-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate was reacted with *S*-(2-aminoethyl)methanesulfonothioate and the *tert*−butyl groups were removed with trifluoroacetic acid. The Gd(III) MTS–ADO3A complex readily formed disulfide bonds with albumin (BSA) in its native and reduced forms and with thiolated silica particles. Four- to five-fold relaxivity increases at 20 MHz were measured on the isolated adducts. The EuMTS-ADO3A chelate was found to be monohydrated by fluorescence and the relaxivity parameters of the Gd(III) complex were obtained by ^{17}O NMR and by measuring the nuclear magnetic relaxation dispersion between 0.01 and 80 MHz. The water exchange time τ_m is increased upon forming disulfide bonds with macromolecules and particles and the relaxivity gains of all the complexes are limited by the τ_m factor. Forming covalent or hydrophobic/electrostatic bonds with BSA seems to bring about similar relaxivity changes but the covalent BSA adducts can be isolated and their properties can be directly studied. The addition of dithiothreitol or glutathione leads to the removal of the metal chelates from the macromolecules, as indicated by the relaxation times reverting to their values before binding. It is thus expected that the chelate will stay in the body long enough for imaging but will still be excreted through the kidneys. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: methanethiosulfonate; albumin; relaxivity; water exchange; nuclear magnetic relaxation dispersion; disulfide link; thiolated silica particles

INTRODUCTION

Gadolinium complexes enjoy wide use as agents for enhancing the contrast of magnetic resonance images because the Gd(III) ion drastically increases the longitudinal relaxation rate $1/T_1$ of the water protons. The water exchange time τ_m and the rotational correlation time τ_r are two important parameters that lead to an increased relaxivity. These parameters can be adjusted by suitable chemical modifications of the Gd(III) complexes in the hope of achieving the largest possible increases of $1/T_1$. For instance, linking a Gd(III) complex to a macromolecule or a particle leads to high relaxivities in the 20–60 MHz range provided the two entities are rigidly bonded to each other so that the small complex acquires the large τ_r value of the slowly rotating macromolecule. This goal can be achieved through a covalent or a noncovalent linkage with synthetic polymers or biological macromolecules (1,2). However, body retention times of covalently linked metal complexes are also lengthened thus exposing the patient to the toxicity of released Gd(III) ions and metabolites (1,3). Preference is thus given to the noncovalent bonding of Gd(III) chelates with macromolecules such as albumin thanks to hydrophobic substituents grafted onto DOTA- or DTPA-type ligands (4–6). While remaining suitable for imaging, the excretion times are sufficiently short to avoid toxicity. The increased contrast is then dependent on the stability constant of the adduct with albumin. Up to a 88% noncovalent bonding has been achieved at a circulating concentration of a DTPA complex of 0.1 mM (6). On the contrary, covalent bonding proved to have a detrimental effect on the clearance of the metal complexes and it was thus suggested to create reversible covalent bonds with macromolecules that are cleaved by endogenous biomolecules or after administration of exogenous substances

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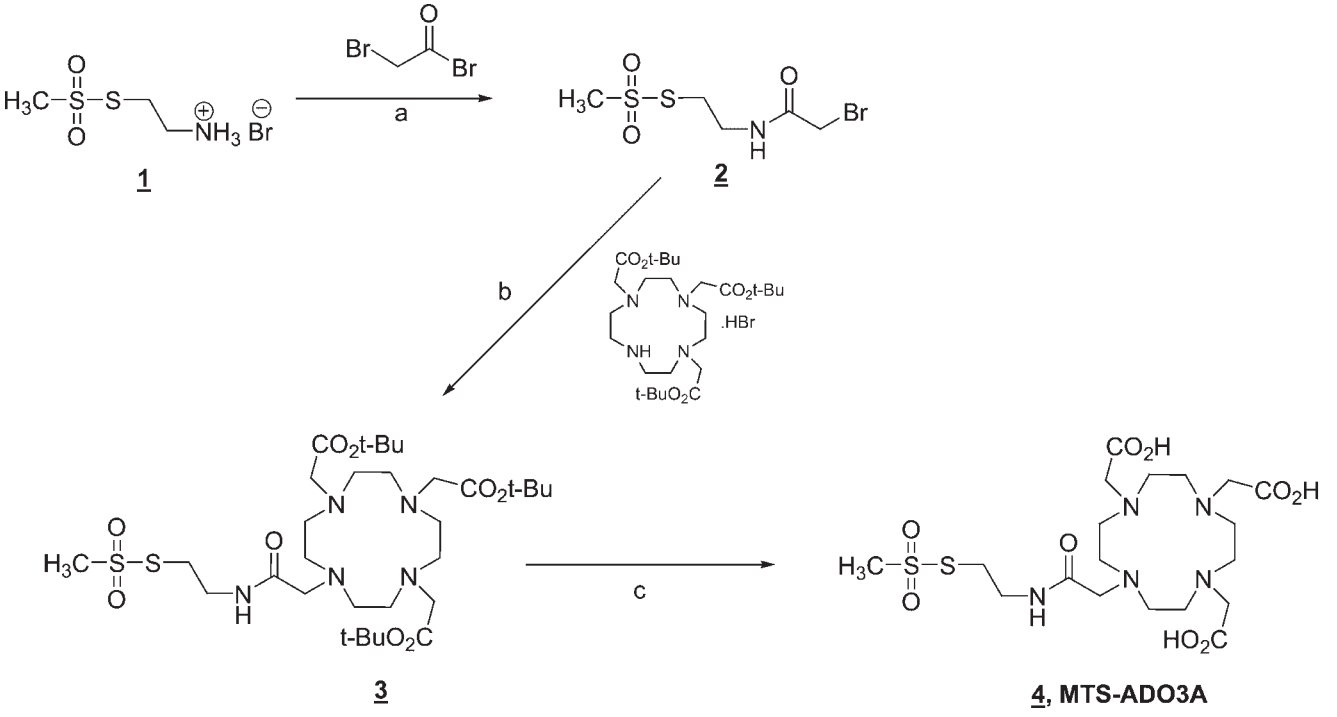
following the imaging examination. The disulfide bond appears particularly interesting in this respect as it is easily cleaved by thiols present in the body (3,7,8). The latter are found in relatively low concentrations in the circulation [about 15 μM (3)] and the residence times in the body of thiolated contrast agents should thus remain sufficiently long to allow imaging. This approach has been adopted by Lu *et al*. (3), who synthesized a DOTA monoamide ligand featuring an amine arm with a disulfide group. The amine function was then reacted with activated carboxylic functions on an L-glutamic acid polymer. After reaction with endogenous thiols, the released chelates are excreted through the kidneys. Similar observations have been made by these authors with DTPA–cystine copolymers (7). The reactivity of the disulfide bond with thiols has recently been exploited by Raghunand *et al.* (8) in a study of the association between human serum albumin (HSA) and a DOTA derivative featuring an amide arm substituted with a propyl or hexylthiol group. The Gd(III) complexes with these ligands partially form a disulfide bond with HSA and the equilibrium between the free and the bonded chelates is modified by the presence of thiols in the reducing environment of tumors. A nearly identical ligand with a shorter aliphatic linker has been reported by Mattila *et al.* (9). The metal-free ligand has been used for tagging phosphopeptides after phosphate elimination in a Michael addition.

The synthesis and properties of the new ligand MTS-ADO3A, **4** (see Scheme 1), featuring a methanethiosulfonate (MTS) function are reported in this paper. Alkylthiosulfonates are known (10,11) to react quantitatively and rapidly with thiol groups under mild conditions. In addition, this reaction is highly selective because amino groups are essentially fully protonated and thus unreactive at physiological pH. It thus becomes possible to isolate the covalent adducts with macromolecules and to investigate directly their relaxation properties and the reactivity of their disulfide bonds with thiols. MTS-ADO3A, **4**, was chosen for this contribution because it is relatively easily synthesized but it is well known that amide arms such as the one it features lengthen the water exchange times of the Gd(III) complexes (6,12). Relaxivity increases brought about by a slow molecular rotation could thus be partially quenched by slow water exchange times. The GdMTS-ADO3A chelate has been reacted with bovine serum albumin (BSA) and thiolated particles, the water exchange times and relaxivities have been measured and the stability of the disulfide bond formed by this complex has been investigated.

RESULTS AND DISCUSSION

Syntheses

The synthesis of MTS-ADO3A, **4**, was accomplished as depicted in Scheme 1. Reagent **1** was obtained as reported in the literature. Methanesulfonyl chloride was reacted with sodium sulfide to prepare sodium methanesulfonothioate (10). The addition of this weak nucleophile to 2-bromoethylamine hydrobromide led to the amine salt **1**. The addition of bromoacetyl bromide led to the as yet unreported compound **2** in good yields. A basic medium was needed to promote this reaction. However, the MTS



Scheme 1. Synthesis of MTS-ADO3A. (a) K_2CO_3, 49%; (b) 1,1,3,3-tetramethylguanidine, 70%, (c) trifluoroacetic acid, 59%.

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group is known to be unstable in basic media. To circumvent this problem, a biphasic system was used and the two reagents were added simultaneously at the same slow rate to the reaction mixture. The crucial step in the synthesis of MTS-ADO3A is the reaction between the methanesulfonothioate **2** and tri-*tert*-butyl 2,2′, 2″-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetate hydrobromide to obtain compound **3**. The macrocyclic reagent in the hydrobromide form was prepared (13) by reacting 1,4,7,10-tetraazacyclododecane with three equivalents of *tert*-butyl bromoacetate in *N*,*N*-dimethylacetamide in presence of three equivalents of sodium acetate that acts as a weak base. The condensation step leading to **3** was carried out without neutralization of the macrocycle hydrobromide in the presence of 1,1,3,3-tetramethylguanidine. Tests proved that this amine was more effective than classical bases such as K_2CO_3, triethylamine or diethylamine. Good yields were achieved provided a small excess of the macrocyclic reagent (0.25 equiv.) was used in order to bring the reaction to completion. This excess was probably needed because of a side reaction between compound **3** and tetramethylguanidine leading to *N*-(bis(dimethylamino) methylene)-2-[2-(methylsulfonylthio)ethylamino]-2-oxoethanaminium as shown by electrospray mass spectrometry. The thiol derivative of **3** was present as an impurity and was successfully removed by a slow elution on a silica gel chromatographic column. Finally, treatment of **3** with trifluoroacetic acid removed the protecting *tert*-butyl groups. As in the previous step, the sought MTS-ADO3A, **4**, was contaminated by its thiol derivative. It was not possible to isolate MTS-ADO3A by the techniques usually employed for purifying polyaminocarboxylic chelates such as recrystallization or chromatography on ion exchange resins. Surprisingly, column chromatography on silica gel yielded the pure compound. The very polar MTS-ADO3A was eluted in rather harsh conditions (butanol–water–acetic acid 1:1:1) while its thiol derivative was retained on the column. MTS-ADO3A was stored at $-18^{\circ}C$ during several months without decomposition.

CONJUGATION OF BOVINE SERUM ALBUMIN WITH GDMTS-ADO3A

The primary structure of albumin is unusual among extracellular proteins as it features a single free sulfhydryl group (Cys-34). The highest degree of substitution that can be achieved by GdMTS-ADO3A on the protein is necessarily lower than one (about 0.6) because approximately 40% of the free sulfhydryl function is oxidized by cysteine and glutathione in circulating plasma (14). In the present work, the average number of metal ions incorporated per protein molecule was determined following gel filtration and exhaustive dialysis. The Gd(III) concentrations in the conjugates solutions were measured by ICP spectrometry and the albumin concentration was assessed by UV absorption at 280 nm or by Lowry's method (15). Both methods led to a number of metal ions per protein molecule of 0.40 ± 0.03 if the molecular weight of 66430 Da was selected for BSA (16) and not to the expected value of 0.60. The single thiol group (Cys34) of BSA is located in a hydrophobic pocket that could not be easily penetrated by GdMTS-ADO3A. This phenomenon has already been reported in the case of the reaction between BSA and Ellman's reagent (17). Moreover, Healy *et al.* (17) reported that the titration of BSA with a 25-fold excess of Ellman's reagent gives only a substitution percentage of 0.51 thiol per BSA. As we used a smaller excess (6.5 equiv.) of GdMTS-ADO3A, a value of 0.40 Gd(III) ions per BSA seems reasonable.

CONJUGATION OF REDUCED BOVINE SERUM ALBUMIN WITH A GD(III) COMPLEX

In addition to a single sulfhydryl group, native albumin also contains 17 cystine residues. To increase the number of metal ions per protein, these disulfide bonds were reduced to thiol functions by treating the protein with dithiothreitol according to the procedure published by Wong *et al.* (18). These authors found that an average of 21 out of 35 available thiol groups could be conjugated. After reaction between reduced albumin and GdMTS-ADO3A, exactly the same conjugation percentage was reached [21 ± 2 Gd(III) per albumin molecule]. As suggested by Wong *et al.* (18), reoxidation of the generated albumin thiols could limit the yield of the conjugation between GdMTS-ADO3A and reduced BSA.

CONJUGATION OF SILICA NANOPARTICLES WITH A GD(III) COMPLEX

The Sicastar® thiolated silica nanoparticles suspensions used in this work contain on average 2.4×10^{13} particles/ml and each particle is covered by about 44 000 thiol functions as determined by titration with Ellman's reagent (19). These particles are nonporous and spherical, their approximate particle diameter is 100 nm and their surface is covered by *O*-propyl-SH groups according to the manufacturer. The average size, shape and morphology of these particles were checked by transmission electron microscopy (TEM). In the scope of this work, the goal was to obtain a sufficiently stable and concentrated suspension to be able to measure relaxation times in well-defined conditions. This aim was achieved by

(1) using lithium hydroxide as a base instead of sodium or potassium hydroxide that are known to decrease considerably the stability of silica suspensions (20);

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(2) discarding the heaviest particles after 3 minutes of centrifugation at 500 rpm.

The longitudinal relaxation times of the suspensions obtained by this procedure did not decrease by more than 3% per hour, which was enough to carry out accurate relaxometric measurements.

RELAXIVITY PROPERTIES

The Gd(III) ion brings about remarkable increases in the experimental relaxation rate $r_{1\mathrm{exp}}$ of the water protons located in its immediate vicinity, either in the first coordination sphere or in the outer sphere (21). The dipolar interaction taking place in the first coordination sphere is accounted for by the Solomon–Bloembergen–Morgan (SBM) equations (22) that are summarized below for a complex GdL:

$$r_{1\,\mathrm{exp}} = \frac{q_{\mathrm{H_2O}}\mathrm{GdL}}{55.56(T_{1\mathrm{M}} + \tau_{\mathrm{m}})} + R_{1\mathrm{outer}} \qquad (1)$$

$$T_{1\mathrm{M}}^{-1} = \mathrm{function}(r^{-6}, \tau_{\mathrm{c}}, \Delta_{\mathrm{t}}, B_0) \qquad (2)$$

$$\tau_{\mathrm{c}}^{-1} = \tau_{\mathrm{r}}^{-1} + \tau_{\mathrm{m}}^{-1} + \tau_{\mathrm{s}}^{-1} \qquad (3)$$

$r_{1\mathrm{exp}}$ expressed in $\mathrm{s}^{-1}\ \mathrm{mM}^{-1}$ is customarily called the relaxivity. The latter depends on a number of factors on the basis of which it is considered that the solution dynamics of a Gd(III)-containing species can be represented reliably by the SBM equations. Among these are $q_{\mathrm{H_2O}}$, the hydration number of the metal ion, the rotational and the solvent exchange correlation times, τ_{R} and τ_{M}, the electronic longitudinal relaxation time, τ_{s}, the correlation time of the modulation of the zero-field splitting, τ_{v}, the mean transient zero-field splitting energy, Δ_{t}, and the metal–proton distance *r*. The contribution of the solvent molecules in the outer coordination sphere $r_{1\mathrm{outer}}$ depends on the metal–proton distance r_{outer} and on the diffusion coefficient D_{diff}. Several of these parameters are unknown and assumptions on their values must be made (23,24) when there is as yet no experimental method to determine their value experimentally. The SBM relaxivity equations have been used extensively for interpreting the dispersion of the nuclear magnetic relaxation rates with the magnetic field (NMRD) of a large variety of magnetic resonance contrast agents (23,24).

The hydration number $q_{\mathrm{H_2O}}$ was deduced from the measurement of the fluorescence lifetimes $\tau_{\mathrm{H_2O}}$ and $\tau_{\mathrm{D_2O}}$ of EuMTS-ADO3A in water and in D_2O using the modified Horrocks equation (25). In this equation, the quenching effect of the amide NH oscillator is taken into account by introducing an additional term (25)

$$q_{\mathrm{H_2O}} = 1.2\left[\left(\frac{1}{\tau_{\mathrm{H_2O}}} - \frac{1}{\tau_{\mathrm{D_2O}}}\right) - 0.25 - 0.075\right] \qquad (4)$$

The values determined for EuMTS-ADO3A and for its mono-conjugate with BSA were 0.9 ± 0.3 and 1.2 ± 0.3 respectively. It is thus assumed that both species are monohydrated. The covalent bonding of the metal chelate with the free cysteine group of BSA does not appear to bring about a decrease in hydration state. Such a decrease has been observed in the non-covalent association between albumin and bis-hydrated Gd(III) complexes of substituted triacetic DOTA-type ligands (26).

The rate of water exchange $1/\tau_{\mathrm{m}}$ of GdMTS-ADO3A has been estimated by ^{17}O NMR. As shown in Fig. 1, the paramagnetic contribution to the transverse relaxation rate of water was measured over the 277–353 K temperature range. The theoretical fitting of the curves was performed assuming $q_{\mathrm{H_2O}} = 1$ and using the Swift and Connick equations (27). The Gd–^{17}O scalar coupling constant (A/h) was fixed at -3.5×10^6 rad s^{-1} in agreement with previous studies (24). Moreover, the activation energy of the modulation of the zero field splitting was fixed at 1 kJ/mol and the temperature dependence of the water exchange time was assumed to exhibit an Eyring dependence (24,28). The calculated values of τ_{m}, ΔH_{m}, τ_{s}, τ_{v}, and Δ_{t}^2 are given in Table 1. It should be noted that Δ_{t}^2 and τ_{v} measured at a single frequency are strongly correlated. As expected for an electrically neutral chelate featuring an amide group, the water molecule coordinated to GdMTS-ADO3A exchanges relatively slowly with the bulk of the solution ($\tau_{\mathrm{m}} = 635$ ns). Similar effects have been noted for triacetic mono- or bisamide linear and cyclic Gd(III) chelates (6,12). It appears that the amide group causes less steric crowding than a carboxylic function in a water exchange dominated by dissociation mechanism (2).

The dispersion of the relaxivity of GdMTS-ADO3A with the frequency at 298 K is presented in Fig. 2. It is a simple S-shape curve between 0.01 and 70 MHz that is very similar to the relaxivity profiles of other small rapidly tumbling complexes such as GdDOTA or

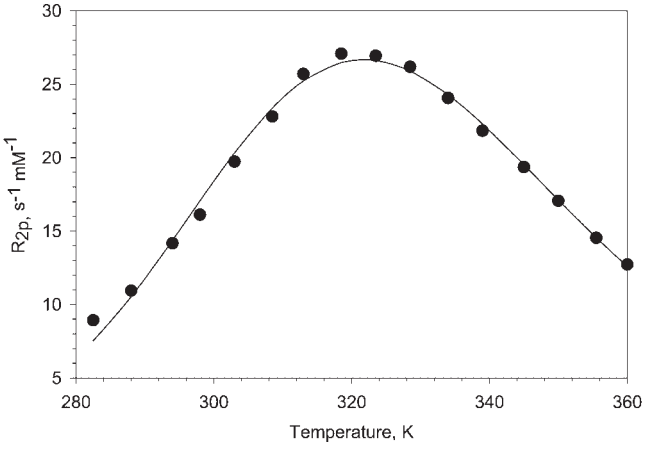

Figure 1. Temperature dependence of the paramagnetic contribution $R_{2\mathrm{p}}$ to the transverse ^{17}O water relaxation rate of GdMTS-ADO3A at 5.87 T, [Gd] = 2.58 mM, pH 5.5.

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Table 1. Relaxivity parameters obtained by best fit fittings of the ^{17}O and NMRD data

Parameter/ligand	Method	$\Delta H^{\neq}$ (kJ mol^{-1})	Δ^2 (10^{20} s^{-2})	τ_v^{298K} (ps)	τ_m^{298K} (ns)	τ_r^{298K} (ps)	D (cm^{-1})
GdMTS-ADO3A	^{17}O NMR	48	$\tau_s \geq 3.3 \times 10^{-10}$ s[a]		622	—	—
GdMTS-ADO3A[b]	NMRD	—	0.15	28	660	98	—
GdMTS-ADO3A[b]	^{17}O NMR + NMRD	48	0.50	10	635	121	—
GdMTS-ADO3A-BSA[c]	NMRD	—	0.07	24	745	~4200[d]	0.026[e]
$(GdMTS\text{-}ADO3A)_{21}$-$BSA_{reduced}$[c]	NMRD	—	0.056	20	770	~4430[d]	0.020[e]
GdMTS-ADO3A-Sicastar[c]	NMRD	—	0.08	19	835	~14 500[d]	0.019[e]

[a]Δ^2 and τ_v are strongly correlated, the best fit was obtained for $\Delta^2 = 0.36 \times 10^{20}$ s^{-2}, $\tau_v = 7$ ps. The value of the electronic relaxation time τ_s is given at low fields, its value increases above $B_0 \approx 0.2$ T. [b]Computed by fitting the NMRD data with the Solomon–Bloembergen–Morgan equations. [c]Computed by fitting the NMRD data with Bertini's computer program for slowly rotating species (32). [d]Uncertain values, the calculated NMRD curves are little influenced by τ_r. [e]Axial component of the zero field splitting (32).

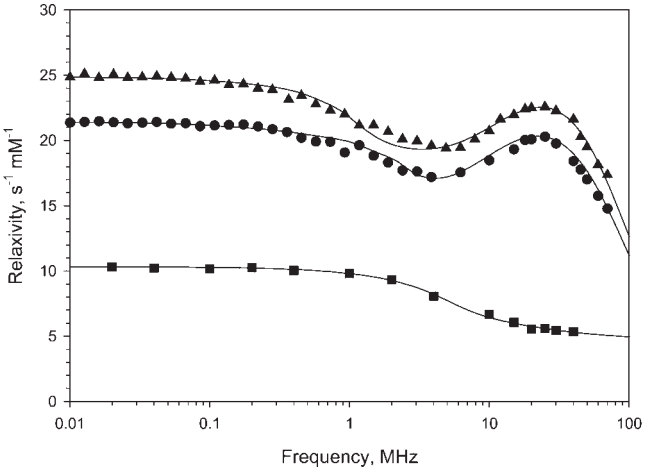

Figure 2. Nuclear magnetic relaxation curves (NMRD) of (■) Gd MTS-ADO3A, (▲) BSA–Gd MTS-ADO3A and reduced BSA–GdMTS-ADO3A (●) conjugate at pH 5.5, 25°C.

GdDTPA (24). Thus, the rotational correlation time τ_r is the major contributor to the relaxivity at all frequencies. The NMRD data were fitted with the SBM equations (24) by taking into account both the outer- and inner-sphere contributions. The metal–water proton distances in the first coordination sphere and in the outer sphere were set at 3.1 and 3.6 Å by reference with literature data (6) and the relative diffusion constant of the complexes was fixed at 2.5×10^{-5} cm^2 s^{-1}(29). As shown in Table 1, the relaxivity parameters were deduced from the experimental data by two methods. First, the fitting of the NMRD curve was performed independently from the ^{17}O measurements using the exchange time τ_m deduced from Fig. 1. In a second approach, the NMRD and ^{17}O experimental data were interpreted simultaneously. The two approaches led to similar values of the rotational correlation times for each complex. The τ_r values of 98–121 ps are close to those previously reported for similar rapidly rotating complexes (24,30). Variations between the Δ^2 values in Table 1 are most probably not physically significant. As mentioned repeatedly in the literature (31), representing adequately the magnetic field dependence of the electronic relaxation time τ_s remains difficult but different combinations of the Δ^2 and τ_v factors have little influence on the calculated water exchange times τ_m.

In contrast with earlier works (4) in which the properties of non-covalent adducts with BSA had to be calculated taking into account the stability constants of the adducts, the relaxivity of the two covalent complexes investigated in the present work is directly obtained from experimental data directly collected on isolated species. The absence of free chelates in the BSA samples was assessed by running the gel permeation chromatography (GPC) and dialysis purification steps reported in the experimental part in presence of the easily detected fluorescent Eu(III) complexes rather than the corresponding Gd(III) species. Furthermore, it was checked that additional dialysis and GPC steps did not change the relaxivity values. The NMRD profiles of the GdMTS-ADO3A and its conjugate with BSA at 298 K are compared in Fig. 2. The NMRD curves of the BSA conjugate display a maximum at 20–40 MHz and high relaxivities at low fields, i.e. two features that are characteristics of slowly rotating species. On formation of a disulfide bond with BSA, the relaxivity at 20 MHz increases from 5.5 to 22.5 mM^{-1} s^{-1}. The relaxivity parameters that account for the shape of the NMRD curves of the BSA conjugate were deduced from a mathematical fit based on the SBM equations that were adapted for slowly rotating paramagnetic complexes in a computer program by Bertini *et al.* (32) for interpreting the NMRD curves of slowly rotating species. Although still approximate, this approach include the static zero field splitting represented by the D factor in Table 1. In keeping with earlier works (33,34), this factor was not taken into account in the interpretation of the NMRD curve of the GdMTS-ADO3A complex as an excellent fitting was obtained with the classical SBM theory and as a detailed EPR investigation would be needed (35). As expected, the rotational correlation time τ_r dramatically increases when GdMTS-ADO3A is covalently bounded

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to BSA and this parameter no longer dominates the relaxivity processes (see Table 1). Moreover, the water exchange time τ_m of GdMTS-ADO3A is increased by about 15% when this compound is coupled to BSA. Although not a general phenomenon (23), a slowing of the water exchange of tetraacetic DOTA-type complexes bounded to BSA or cyclodextrins has already been reported by Aime *et al.* (4,36) and was attributed to the presence of an ordered water layer adsorbed on these polymeric structures. Because of a network of hydrogen bonds, the water exchange rate would be slower than expected.

It should be noted that the phenomena observed here with covalently bounded Gd(III) chelates are similar to those already reported for chelates simply associated with BSA by hydrophobic/electrostatic interactions. The lengthening of the water exchange times does not seem to depend on a preferential orientation of the metal chelates on the protein surface (5) that would be caused by the formation of a disulfide bridge with cysteine. Also noteworthy is the rather low value of Δ_t^2. Similar low values have been reported in studies on albumin adducts by authors who used the computational procedure used here (37) or deliberately limited the fit to the 5–50 MHz range (6,38). The magnitude of Δ_t^2 remains a subject of debate (35,39,40).

Close to 21 GdMTS-ADO3A complexes were found to be covalently bounded to the reduced form of BSA but the relaxivity curve is close and quasi parallel to the 1:1 Gd MTS-ADO3A-BSA compound (Fig. 2). As expected, the two NMRD curves are accounted for by similar parameters. On a per Gd(III) ion basis, the number of complexes fixed on BSA does not seem to influence the longitudinal relaxivity.

The NMRD profile of GdMTS-ADO3A covalently linked to thiolated Sicastar® silica particles is presented in Fig. 3 and the relaxivity parameters deduced from mathematical fits are included in Table 1. As already observed for adducts with BSA, the water exchange time τ_m of GdMTS-ADO3A is increased upon formation of disulfide bonds with silica particles. The calculated rotational correlation times τ_r are very high but the relaxivity gains remain modest because they are limited by the slow exchange time. The main interest of these systems is probably not the relaxivity obtained per Gd(III) but the relaxivity r_1 obtained per particle which could be estimated at $190\,000\ s^{-1}\,mM^{-1}$ (at least 10 000 Gd per particle as determined by ICP). However, their tendency to sediment with time remains an unsolved problem and smaller particles will have to be tested. Relaxivities per particle of the order of $160\,000\ s^{-1}\,mM^{-1}$ have been reported for superparamagnetic nanoparticles (41).

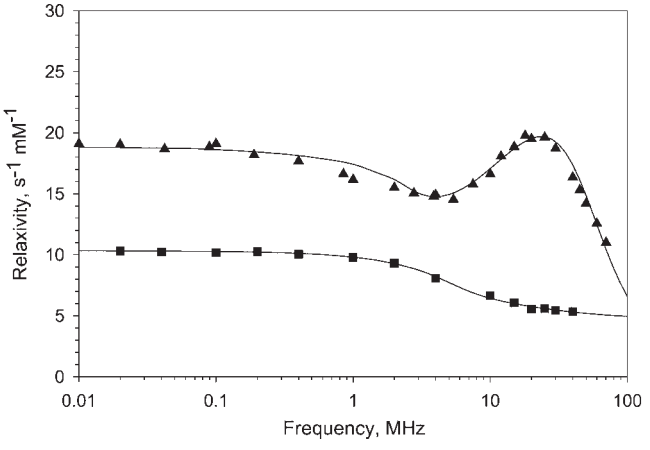

Figure 3. Nuclear magnetic relaxation curves (NMRD) of (■) Gd MTS-ADO3A, (▲) Sicastar®–Gd MTS-ADO3A conjugate at pH 5.5, 25°C.

REACTIVITY OF THE DISULFIDE BOND IN THE BSA–GDMTS-ADO3A CONJUGATE

The reactivity of the disulfide bond formed by GdMTS-ADO3A was tested in presence of dithiothreitol (DTT) as illustrated in Scheme 2. This reagent is well known to completely reduce disulfide links (42). Iodoacetamide was added to the reaction mixture in order to prevent the gelation of BSA thanks to the blockage of the released thiol functions (43). DTT was added in excess (50 equiv.) to the BSA–GdMTS–ADO3A conjugate and the relaxivity changes with time were measured. The relaxivity decreased from 22 to $6.5\ mM^{-1}\,s^{-1}$ following the addition of DTT. As this value is very close to the relaxivity of GdMTS–ADO3A in water, it is concluded that all the Gd(III) ions in the sample were indeed covalently linked to BSA by a disulfide bond. The stability of this bond was also tested in the presence of glutathione (GSH) as shown in Scheme 2. This tripeptide is the most abundant reducing molecule in the circulation (44) and is also the most abundant non-proteinaceous thiol in mammalian cells (45). The reaction between disulfide links and GSH leads to the release of the gadolinium chelate through a thiol–disulfide exchange as also observed for a gadolinium complex featuring a free thiol function in the presence of homocysteine (8). The relaxation time changes of a solution of the reduced BSA conjugate with GdMTS–ADO3A upon the addition of 100 equivalents of GSH follow the pseudo-first-order reaction kinetics $T_1 = a + be^{-kt}$ with $k = 1.6 \times 10^{-5}\ s^{-1}$ in the experimental conditions of Fig. 4 (BSA –GdMTS-ADO3A conjugate 0.24 mM, 20 MHz, 25°C, after addition of 100 equiv. of glutathione).

CONCLUSIONS

There are two examples of MTS containing DTPA ligands in the literature. Two acetate arms of DTPA have been substituted with *S*-(2-aminoethyl) methanesulfonothio-

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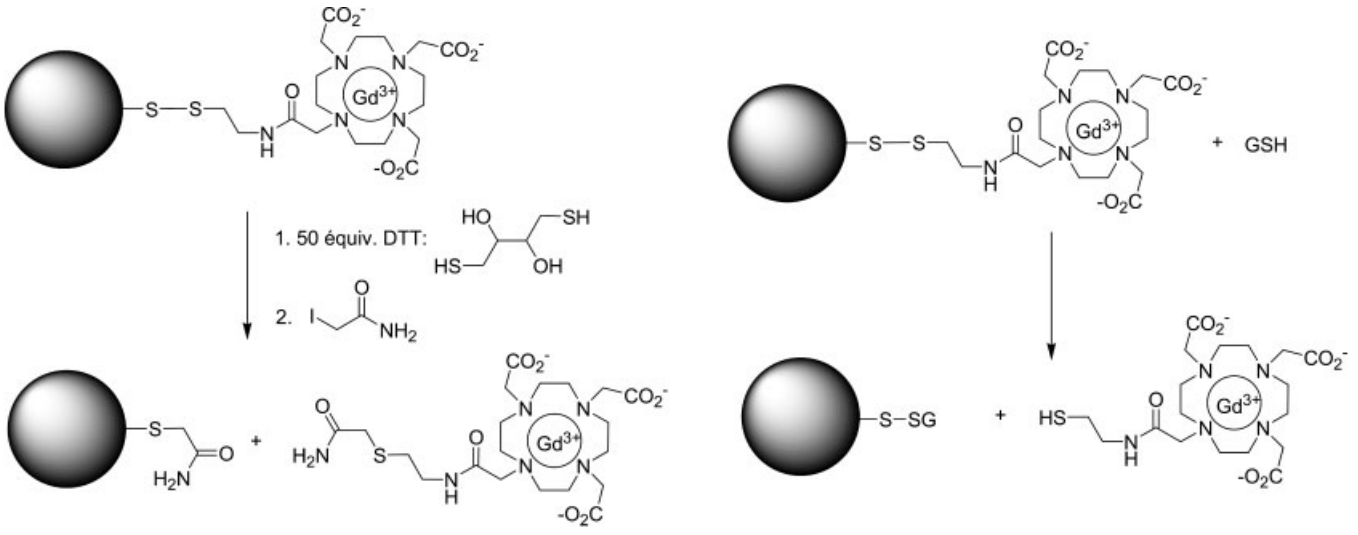

Scheme 2. Reaction of the BSA–GdMTS-ADO3A conjugate with dithiothreitol (left) and glutathione (right).

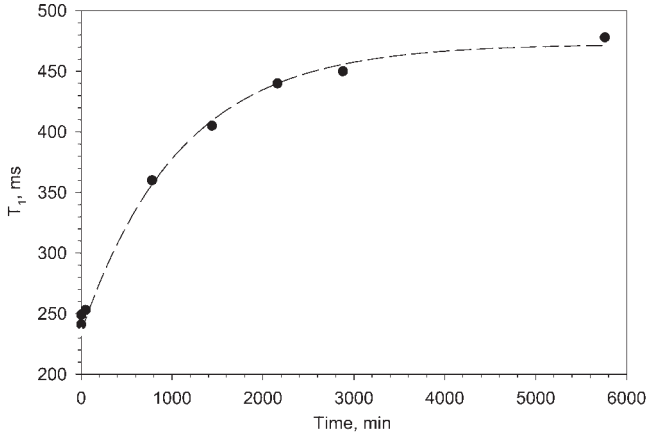

Figure 4. Evolution of the longitudinal relaxation time T_1 of a 0.24 mM solution of GdMTS-ADO3A–BSA at 20 MHz, 25°C, following the addition of 100 equivalents of glutathione.

ate, **1**, to form a bis-amide ligand whose Yb(III) complex was doubly attached to a protein in order to unravel its solution structure by NMR (46). Reagent **1** was also added to one of the carboxylic function of a DTPA ligand substituted with a carbostyril unit so as to form reactive fluorescent lanthanide chelates (47). To the best of our knowledge, the present contribution is the first to report on the synthesis and the relaxivity properties of a bifunctional ligand of the DOTA-type featuring a methanethiosulfonate group (MTS). The DOTA structure was chosen in this report because it forms kinetically inert and thermodynamically highly stable Gd(III) complexes (1) and the MTS function was selected because of its high reactivity towards thiols and thus the possibility of forming quantitatively covalent disulfide bonds with macromolecules or particles. In addition, the disulfide bonds is unstable *in vivo*, thus ensuring that the Gd(III) complexes could be excreted from the body while leaving enough time for magnetic resonance imaging. Well planned pharmacokinetic studies would be needed to completely unravel the *in vivo* stability of the BSA-GdMTS-ADO3A conjugate but the kinetic data presented in Fig. 4 seem to indicate that the small amount of free thiols in the circulation is not sufficient to cause a significant degradation of the disulfide bond in the conjugate within a reasonable length of time. An injection or the ingestion of glutathione would be necessary to achieve a complete degradation. Glutathione is known for its low toxicity (LD50 >2000–5000 mg/kg in mouse) and is used as a nutritional supplement in humans (3).

The MTS-ADO3A ligand, **1**, is readily synthesized but its water exchange time τ_m is long, as shown by ^{17}O NMR, and hence the relaxivity is partially quenched in keeping with earlier studies that shows that replacing a carboxylic function by an amide group slows down the water exchange rate (1,2). GdMTS-ADO3A was reacted with the single free cysteine of BSA, with reduced BSA and with thiolated silica nanoparticles. The relaxivity is increased by a factor of 4–5 at 20 MHz. Although sizeable, this increase is not as high as hoped because the favorable increase in rotational correlation times τ_r is partially compensated by an unfavorable lengthening of the water exchange times τ_m. This effect has already been reported for Gd(III) chelates linked to albumin by non-covalent bonds and has been assigned to stable hydrogen-bonded layers of water molecules on the macromolecule surface (4–6). This hypothesis could also account for the relaxivity differences observed between BSA and silica particles loaded with Gd(III) complexes. Although the rotational correlation times τ_r are considerably longer for the Sicastar particles, the water exchange times τ_m are also longer and hence the relaxivities are lower, possibly because of the strong adsorption of water molecules on unprotected silanol groups.

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MATERIAL AND METHODS

General

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX 400 spectrometer and chemical shifts (δ) are reported in parts per million relative to tetramethylsilane. Nuclear magnetic relaxation dispersion (NMRD) data were collected from 0.01 to 70 MHz on an upgraded Stelar Spinmaster FFC2000 relaxometer (Stelar, Mede, PV, Italy) to which a Bruker 2 T permanent magnet had been connected. ^{17}O-NMR measurements were carried out on 2.58 mM (pH = 5.5) solutions of metal complexes. ^{17}O-enriched water (20% $H_2^{17}O$, Chemotrade, Leipzig) was added to the solutions of metal complexes in order to reach a 1.5% ^{17}O enrichment. The ^{17}O transverse relaxation times were measured using the standard Carr–Purcell–Meiboom–Gill spin echo pulse sequence on a Bruker Avance AM250 spectrometer. Luminescence lifetimes were measured on a Photon Technology International (PTI, Birmingham, NJ, USA) spectrometer equipped with a GL-3300 nitrogen laser using 2′[1,1′-biphenyl]-4-yl-6-phenylbenzoxazole (PBBO dye) to excite the Eu^{3+} ion at 394 nm. The recorded luminescence decay curves (emission monitored at 592 nm) were fitted to a mono-exponential function using the PTI Time Master software. Inductively coupled plasma analyses (ICP) were performed on a Bausch & Lomb ARL 3510 spectrometer. Electrospray mass spectra were obtained on Fisons VG platform (ES-MS) and Bruker Daltonics micrOTOF spectrometers (TOF-ES-MS). Thiolated Silica nanoparticles suspensions (Sicastar-SH, polydispersity index <0.25, size 100 nm) were purchased from Micromod Partikeltechnologie GmbH, Rostock, Germany (http://www.micromod.de/). Transmission electron microscopy (TEM) images were recorded with a Philips CM100 microscope equipped with a Gatan 673 CCD camera, and transferred to a computer equipped with the Kontron KS100 system. Samples were prepared by dipping a Formvar-coated copper grid into the nanoparticles suspension. Complexometric titrations (48) with ^{60}Co were carried out on an InstantImager autoradiography system (Canberra Packard, USA).

S-2-(2-bromoacetamido)ethyl methanesulfonothioate, 2

S-(2-aminoethyl)methanesulfonothioate hydrobromide (10), **1**, (2.60 g, 11 mmol) was dissolved in water (5 ml) and placed in a 100 ml round-bottomed flask. This solution was cooled in an ice bath and stirred vigorously. Solutions of bromoacetyl bromide (2.67 g, 13.2 mmol) in 10 ml of dichloromethane and potassium carbonate (3.34 g, 24.2 mmol) in 10 ml of water were added at the same rate from two dropping funnels. After 1 h, the reaction mixture was allowed to reach room temperature and was extracted three times with 25 ml with dichloromethane. The organic fractions were combined, dried over $MgSO_4$ and finally evaporated *in vacuo* to yield 1.5 g of compound **2** as a yellow oil. This compound was used without further purification in the next synthetic step. Yield 49%. ^{1}H NMR ($CDCl_3$) δ: 7.14 (s br, 1H), 3.68 (s, 2H), 3.66 (m, 2H), 3.37 (s, 3H), 3.34 (t, 2H, J = 6 Hz) ppm. ^{13}C NMR ($CDCl_3$) δ: 167.4, 51.4, 40.6, 36.1, 29.7 ppm.

Tri-tert-butyl 2,2′,2″-(10-{2-[2-(methy lsulfonylthio)ethylamino]-2-oxoethyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate, 3

Tri-*tert*-butyl 2,2′,2″-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate hydrobromide (13) (0.665 g, 1.11 mmol) was added under argon to a mixture of 1,1,3,3- tetramethylguanidine (0.384 g, 3.33 mmol) and compound **2** (0.458 g, 1.66 mmol) dissolved in 10 ml of dry dimethylformamide. The reaction mixture was stirred at room temperature for 72 h. Brine (30 ml) was added and the resulting solution was extracted three times with 25 ml of ethyl acetate. The organic fractions were combined, dried over $MgSO_4$ and evaporated *in vacuo*. The brown oily residue obtained was loaded on a silica gel column that was washed first with EtOAc–MeOH 9:1 followed by EtOAc–MeOH 5:1 and finally with EtOAc–MeOH 1:1 to elute the sought product. The fractions containing the pure product as indicated by ES-MS were evaporated and dried *in vacuo* to obtain 0.552 g of a solid white foam. Yield 70%. ES-MS: *m/z* 710 $(M+H)^+$, 732 $(M+Na)^+$, 355 $(M+2H)^{2+}/2$. ES-TOF-MS: calcd for $C_{31}H_{59}N_5Na\ O_9S_2$ $(M+Na)^+$ 732.3646, found 732.3629. ^{1}H NMR ($CDCl_3$) δ: 8.59 (br s, 1H), 3.50–1.90 (m, 31H), 1.24 (s, 27H) ppm. ^{13}C NMR ($CDCl_3$) δ 173.4, 173.3, 173.1, 83.0 (2C), 57.1, 56.6 (2C), 52.3, 53–47 (br, 4C), 38.9, 37.1, 29.0, 28.9 ppm.

2,2′,2″-(10-{2-[2-(methylsulfonylthio) ethylamino]-2-oxoethyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid, MTS-ADO3A, 4

Triester **3** (1.0 g, 1.41 mmol) was dissolved in 20 ml of a 1:1 CH_2Cl_2–trifluoroacetic acid mixture. The solution was stirred at room temperature for 18 h. Evaporation of the solvents under reduced pressure yielded a viscous orange oil. This residue was purified by column chromatography on silica gel. For this purpose, the oil was dissolved in a butanol–methanol mixture and was loaded onto the column. After washing with butanol, butanol–water–acetic acid 4:1:1, the sought product was finally eluted with butanol–water–acetic acid 1:1:1. The fractions containing only the desired product were

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combined to provide 0.450 g of compound **1** as a solid white foam. The purity of MTS-ADO3A was checked by a complexometric titration (48) with ^{60}Co on an InstantImager autoradiography system (Canberra Packard, USA) and by a colorimetric assay of the MTS function (49). Samples obtained in different syntheses contained varying amounts of dissolved silica (5–20%) that was eliminated by gel permeation chromatography after coupling the ligand with macromolecules. An analytically pure sample was obtained by ion exchange chromatography (AG1-X2, formic acid gradient). Yield 59%. ES-MS *m/z* 542 $(M+H)^+$, 564 $(M+Na)^+$, 272 $(M+2H)^{2+}/2$. ES-TOF-MS: calcd for $C_{19}H_{36}N_5O_9S_2$ $(M)^+$ 542.1949, found 542.1948. ^1H NMR (D_2O) δ: 4.0–2.0 (br m). ^{13}C NMR (D_2O) δ: 176.9, 174.3, 172.7, 58.9, 57.5, 56.2, 53.9, 53.4, 52.5, 51.2, 50.9, 41.2, 37.8 ppm.

Preparation of gadolinium complexes

MTS-ADO3A, **1** (17.5 mg, 0.032 mmol), was dissolved in water (5 ml) and 192 μl of a 0.167 M aqueous solution of $GdCl_3$ (0.032 mmol) was added under stirring. The pH of the solution was continuously maintained at 5.5 by the addition of a NaOH 0.1 M solution until no more pH variation was observed. This solution was used immediately without further treatment to prepare the DOTA-conjugated macromolecules.

Studies of the properties of the GdMTS-ADO3A chelate were performed on solutions purified by elution with water on a column of Chelex 100 resin (Sigma). The free metal ions were fully retained on the column and no decomposition of the complex was observed. The Gd^{3+} concentrations were determined by ICP analysis.

Synthesis of a conjugate between BSA and GdMTS-ADO3A

Typically, bovine serum albumin (1.08 g of a 30% aqueous solution, 0.0049 mmol) was dissolved in 20 ml of a 3-(*N*-morpholino)-propanesulfonic acid buffer (20 mM, KCl 150 mM, EDTA 1 mM, pH 7.5) and added to an aqueous solution of GdMTS–ADO3A (0.032 mmol). This reaction mixture was stirred at room temperature for 3 h. The solvent was evaporated in a low vacuum SpeedVac concentrator. The residue was dissolved in a minimum of water and loaded onto a Sephacryl S-100 column (1.5×40 cm). The column was eluted with water (0.5 ml/min, monitored by UV absorption) and the fraction displaying the most intense signal was concentrated to give a solution of paramagnetic conjugate. The Gd^{3+} concentrations were determined by ICP analysis after digestion of the evaporated solutions in boiling HNO_3. It was verified that a dialysis after the gel filtration step did not change the relaxivity.

Synthesis of a conjugate between reduced BSA and GdMTS-ADO3A

Bovine serum albumin (0.140 g of a 30% aqueous solution, 0.00063 mmol) was dissolved in 10 ml of a tris(hydroxymethyl)aminomethane hydrochloride buffer (0.2 M, pH 8). This solution was made 6 M in guanidine hydrochloride and 0.1 M in dithiothreitol and was stirred 2 h at room temperature. The pH was brought to 3 with glacial acetic acid and the solution was dialyzed twice against 1 l of 0.1 M aqueous acetic acid solution at 4°C for 12 h (cellulose membrane, diameter 6 mm, Aldrich D9277). The dialyzed solution was filtered through a Millipore filter (45 μM) and was made up to 6 M in guanidium hydrochloride and 50 mM in ammonium carbonate at pH 8. Solid EDTA in the acid form (0.020 mmol) and GdMTS-ADO3A (0.043 mmol) were finally added. The reaction and the purification were carried out as described above for GdMTS-ADO3A-BSA.

Synthesis of GdMTS-ADO3A-Sicastar particles

A solution of GdMTS-ADO3A (9.6 μmol) was prepared exactly as mentioned above except that LiOH was used instead of NaOH. A 400 μL aliquot of a 5 mM DTPA solution whose pH was brought to 5.5 with a 0.06 M LiOH solution was added to the GdMTS-ADO3A solution. This mixture was finally poured into 1 ml of a suspension of Sicastar thiolated silica nanoparticles suspension ($\sim 2.4 \times 10^{13}$ particles/ml according to the supplier). The pH was maintained close to 7.5 with diluted LiOH solutions and the mixture was stirred overnight. The suspension was centrifuged at 2500 rpm for 1 h. The supernatant was discarded and the remaining material was suspended in 10 ml of an acetic acid/lithium acetate buffer (pH 5.5, 0.1 M). This operation was repeated five times to eliminate unbound chelates. Finally, the suspension was centrifuged at 500 rpm for 3 min. The solid fraction was eliminated and the suspension was used as such for relaxometry studies. This procedure leads to suspensions that are stable for several hours. The gadolinium content was determined by ICP after treatment with hydrogen fluoride.

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