FULL PAPER

In vitro Relaxometric and Luminescence Characterization of P792 (Gadomelitol, Vistarem®), an Efficient and Rapid Clearance Blood Pool MRI Contrast Agent

Luce Vander Elst,[a] Isabelle Raynal,[b] Marc Port,[b] Pierre Tisnès,[c] and Robert N. Muller*[a]

P792 (Gadomelitol, Vistarem®), a hydrophilic high molecular weight ($MW = 6473$ g/mol) derivative of DOTA-Gd is a rapid clearance blood pool agent (RCBPA) characterized by high proton relaxivities r_1 and r_2 at 310 K in the current range of clinical imaging magnetic fields. Luminescence data of the Eu^{III} complex, as expected for this type of DOTA derivative, agree with the presence of one water molecule in the first coordination sphere of the lanthanide ion. The stability of the water proton longitudinal relaxation rate in the presence of zinc(II) and phosphate ions showed that the transmetallation by Zn^{II} ions is negligible. The temperature dependence of the water oxygen-17 transverse relaxation rate gave a τ_M smaller than 100 ns at 310 K. No significant increase of longi-

tudinal or transverse relaxation rates in serum or HSA solution was noticed, precluding association with high molecular weight blood components. The proton Nuclear Magnetic Relaxation Dispersion (NMRD) profile of the water solution showed a maximum of longitudinal relaxivity between 20 and 40 MHz ($r_1 \approx 40$ s^{-1}mM^{-1} at 310 K). Fitting of the proton NMRD curve by the classical outersphere and innersphere models, including or not additional second sphere water molecules, gave a rotational correlation time of approximately 2 to 3 ns and a large value of the electronic relaxation time at low field ($\tau_{SO} \approx 500$ ps).
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Introduction

The development of new paramagnetic MRI contrast agents with high relaxivity usually involves the decrease of their rotational mobility by covalent or non-covalent binding to endogenous or exogenous macromolecules.[1–5] The resulting bulky contrast agents are confined in the vascular space and their renal excretion is reduced. Recently, medium molecular weight ($MW > 5000$ g/mol) contrast agents based on the DOTA-Gd structure tetra-substituted by hydrophilic arms have been developed. For example, P760 ($MW = 5.3$ kDa) is a low diffusion agent which extravasates at a slow rate through the vascular endothelium but is freely excreted by the kidneys.[6–8] Due to a long rotational correlation time ($\tau_R \approx 2$ ns at 310 K) its proton relaxivity is quite large, with a maximum around 25 s^{-1}mM^{-1} observed between 0.35 and 1.4 T.[9]

Another macromolecular DOTA-Gd structure, P792 (Gadomelitol, Vistarem®, $MW = 6.47$ kDa) has proved also

to be a rapid clearance blood pool agent (RCBPA) (Figure 1) with a limited diffusion across the normal endothelium.[6,8] It is mainly excreted by glomerular filtration and has been reported to be well suited for high-quality first-pass and equilibrium-phase MR angiography,[10] visualization of the coronary arteries and their branches,[11] assessment of viability of infarcted myocardium,[12] differentiation of tumor-bearing lymph nodes from reactive inflammatory and normal nodes,[13] and for the distinction between metastatic and relatively benign cancers.[14] A phase I clinical trial has established its safety in humans.[15] In a previous study,[8] some physicochemical parameters were reported like its high proton relaxivity at 20, 40, and 60 MHz and at body temperature. This work aims at extending these preliminary data with a comprehensive in vitro physicochemical characterization encompassing luminescence and oxygen-17 relaxometry and an investigation of the possible non-covalent interaction with blood proteins as well as the analysis of the proton NMRD profile and the determination of related physical parameters.

Results and Discussion

Luminescence of the Europium Complex

According to Horrocks and Sudnick,[16] the difference in the decay rate of Eu^{3+} luminescence in H_2O ($1/\tau_{H_2O(ms)}$) and

[a] Department of Organic and Biomedical Chemistry, NMR and Molecular Imaging Laboratory, University of Mons-Hainaut, 7000 Mons, Belgium
Fax: +32-65-37-35-20
E-mail: robert.muller@umh.ac.be
[b] Guerbet, Research Department, 95943 Roissy Cdg Cedex, France
[c] Laboratoire de Synthèse et Physicochimie de Molécules d'Interêt Biologique / UMR 5068, Université Paul Sabatier, 118 route de Narbonne 31062 Toulouse Cedex 4, France
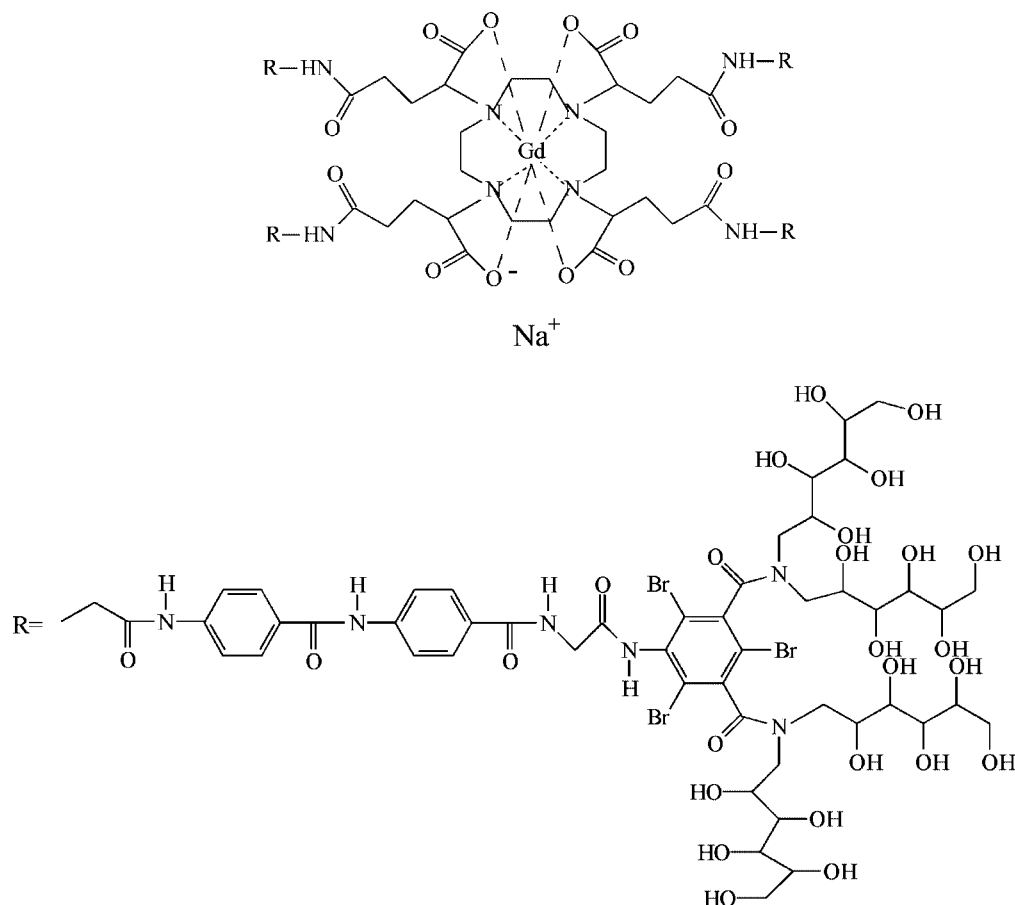
Figure 1. Chemical structure of P792.

D_2O ($1/\tau_{D_2O(ms)}$) solutions depends on the number of water molecules (q) in the first coordination sphere of the complex [Equation (1)]. More recently, Beeby et al.[17] included in the relevant equation the contribution from water molecules diffusing close to the Ln^{3+} complex and from other protons behaving as exchangeable oscillators [Equation (2)]. The measured luminescence lifetimes of P792-Eu in H_2O and D_2O were 0.71 ms and 2.32 ms, respectively. The corresponding calculated q values are 1.02 using Horrock's equation or 0.87 according to Beeby et al.

$$q = 1.05 \cdot \left(\frac{1}{\tau_{H_2O}} - \frac{1}{\tau_{D_2O}} \right) \qquad (1)$$

$$q_{corr} = 1.2 \cdot \left(\frac{1}{\tau_{H_2O}} - \frac{1}{\tau_{D_2O}} - 0.25 \right) \qquad (2)$$

where τ is the lifetime (in ms) of the complex in H_2O and D_2O.

These values agree with the q value reported for the precursor P730.[18,19] As expected, the Gd^{III} ion is thus coordi-

nated to the four nitrogen atoms of the macrocycle, the four carboxylate groups, and one water molecule.

Oxygen-17 Relaxometry of the Gadolinium Complex

The residence time of the coordinated water molecule is a very important parameter likely to limit the innersphere relaxivity of gadolinium complexes and more particularly of bulky structures. In such complexes, the expected enhancement in relaxivity resulting from the slow tumbling of the complex can indeed be quenched by a too slow water exchange.

It is well established that the exchange rate of the water molecule in the first coordination sphere of Gd^{3+} can be determined by analyzing the temperature dependence of the paramagnetic transverse relaxation rate ($R_2p = 1/T_2p$) of the oxygen-17 NMR signal of the aqueous solution.[20−22] The temperature dependence of ^{17}O R_2 was, therefore, studied from 290 K to 357 K (Figure 2). Lower temperatures were not investigated to avoid problems related to an increase in viscosity. The qualitative analysis of the transverse relaxation rate data indicates that the water exchange

is quite fast. Moreover, the much larger values obtained for the longitudinal relaxation rates of P792 as compared to those of DOTA-Gd[22] agree with a complex of higher molecular weight.
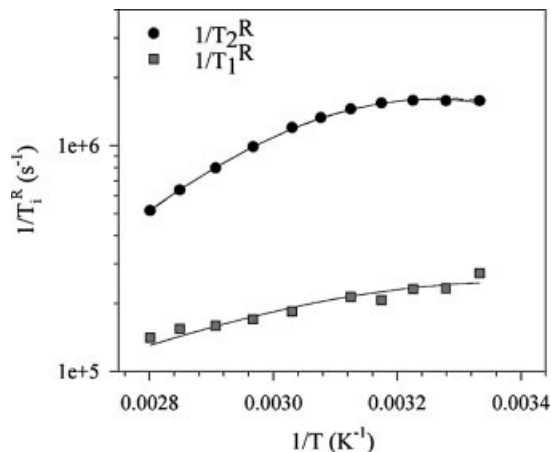

Figure 2. Reduced transverse $\{1/T_2R = 55.55/(T_2p*[\text{Gd complex}])\}$ and longitudinal ^{17}O relaxation rate of water in P792 solution as a function of the reciprocal of temperature.

Quantitative analyses of the experimental data were performed with or without quadrupolar contribution, leading to 2 sets of values (Table 1). The first fitting of the experimental data was performed on the transverse relaxation rates with a model where the transverse relaxation rate of the bound nuclei of ^{17}O ($1/T_{2M}$) results mainly from scalar interaction with the gadolinium ion as described in ref.[20]. The second fitting was performed simultaneously on the transverse and longitudinal relaxation rate data with a model taking into account quadrupolar and dipolar contributions for $1/T_{1M}$ and quadrupolar and scalar contributions for $1/T_{2M}$.[9]

Table 1. Parameters obtained from the fitting of the transverse and longitudinal relaxation rates of oxygen-17.

	Without quadrupolar contribution	With quadrupolar contribution
τ_V^{298} [ps]	11.8 ± 0.3	12.8 ± 1.1
B [10^{20} s^{-2}]	4.06 ± 0.10	3.45 ± 0.41
E_v [kJ/mol]	0.9 ± 44.0	1.4 ± 1.5
$2\pi A/h$ [10^6 rad s^{-1}]	-4.46 ± 0.03	-3.95 ± 0.36
$\Delta H^{\#}$ [kJ/mol]	46.3 ± 0.09	46.9 ± 0.3
$\Delta S^{\#}$ [J/mol·K]	40.3 ± 1.3	40.6 ± 0.3
τ_M^{310} [ns]	79.6 ± 5.0	95.7 ± 14.9
τ_R^{298} [ns]		3.2 ± 0.2
τ_R^{310} [ns]		2.4 ± 0.2
E_R [kJ/mol]		17.0 ± 0.2
χ [MHz]		6.60 ± 0.35

The following parameters are simultaneously determined:
i) The hyperfine coupling constant between the oxygen-17 nucleus and the electronic spin of Gd^{3+} ($2\pi A/h$);
ii) the parameters related to the electronic relaxation times (τ_{S1} and τ_{S2}), i.e. the correlation time for the modulation of the zero field splitting (τ_V), the activation energy for this process (E_V), and B which depends on the trace of the

square of the zero field splitting tensor (Δ^2) and on the electron spin S ($B = \Delta^2\{4S(S + 1) - 3\}/25$);
iii) the parameters of the water exchange rate, i.e. the enthalpy ($\Delta H\neq$) and the entropy ($\Delta S\neq$) of activation;
iv) the rotational correlation time τ_R and the activation energy for the rotation (E_R), as well as a term related to the quadrupolar coupling constant and the asymmetry parameter of ^{17}O ($\chi = [1 + \eta^2/3]^{1/2}[2\pi e^2qQ/h]$) when the quadrupolar contribution is taken into account.

The fitted value of τ_M obtained with the first model is approximately 80 ns at 310 K, a value close to that of the parent complex DOTA-Gd ($\tau_M^{310} \approx 97$ ns[22]). When the quadrupolar contribution is taken into account, the data from both longitudinal and transverse relaxation rates were fitted simultaneously and the distance between the gadolinium ion and the oxygen nucleus of the coordinated water molecule was set to 0.25 nm.[22] The fitted value of τ_M^{310} was then slightly larger (≈ 96 ns) and the τ_R^{310} value was equal to 2.4 ns, a value slightly larger than that obtained for P760 a compound of rather similar size and shape.[9] Such values of τ_M^{310} although not optimal to ensure a maximum relaxivity are not too large and, if any, their influence on the proton relaxivity should be small or moderate.

Transmetallation by Zn^{2+} Ions

The suitability of paramagnetic complexes as MRI contrast agents depends not only on their relaxivity but also on various other factors, among which is their inertness towards transmetallation by endogenous ions like Zn^{2+}. This can be assessed in vitro by H-1 relaxometry since, as already reported, transmetallation of gadolinium complexes by Zn^{2+} ions induces a release of Gd^{3+} ions which are trapped in a buffered solution containing phosphate ions. The subsequent decrease of the water proton relaxation rate quantitatively reflects the extent of the transmetallation process.[23] In the context of this protocol, the relaxation rate of macrocyclic gadolinium complexes like DOTA-Gd, HPDO3A-Gd [tris(carboxymethyl)-10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane gadolinium(III)], and P760 does not vary, whereas R_1p drops to about 53% of its initial value for DTPA-Gd, 18% for DTPA-BMA-Gd, and 85% for MS-325 after 1900 minutes showing that a significant transmetallation takes place for these open-chain gadolinium complexes.[9,23,24] For P792, lower concentrations of the complex and of Zn^{2+} were used (0.8 mM and 0.87 mM, respectively, instead of 2.5 mM in the original protocol). The data were thus compared to those obtained for DTPA-Gd at identical concentration ([DTPA-Gd] = 0.8 mM and [Zn^{2+}] = 0.87 mM). No significant evolution of the proton paramagnetic relaxation rate was observed for P792 over a period of more than 3000 minutes. This agrees with the absence of transmetallation by Zn^{2+} ions. In the same conditions, the R_1p of DTPA-Gd decreased by about 60% during the same period (Figure 3). These data confirm the very high kinetic and thermodynamic stabilities of DOTA-like

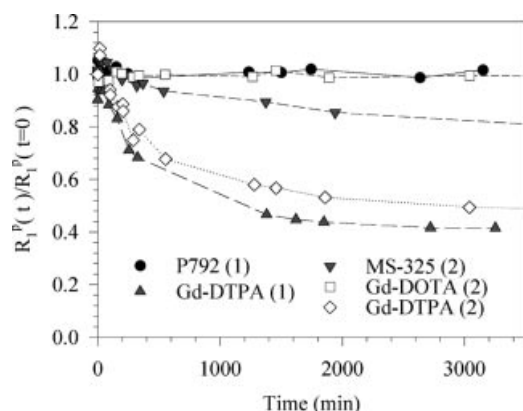complexes of gadolinium as compared to DTPA-like complexes.[23,24]

Figure 3. Evolution of the ratio $R_1p(t)/R_1p(t = 0)$ with time for solutions containing $ZnCl_2$ and P792 (circles), DTPA-Gd (up triangles and diamonds), MS-325 (down triangles), DOTA-Gd (squares). The experimental conditions are: (1): $ZnCl_2$ (0.87 mм) and complex-Gd (0.80 mм) at pH 7.0 and 37 °C ($B_0 = 0.47$ T) and (2): $ZnCl_2$ 2.5 mм and complex-Gd (2.5 mм) at pH 7.0 and 310 K ($B_0 = 0.47$ T).

Proton NMRD Profile

The proton longitudinal relaxation rates were measured at 310 K between 0.01 MHz and 300 MHz on 0.285 mм and 1 mм solutions of P792; the transverse relaxation rates were measured at 20 MHz, 60 MHz and 300 MHz. The calculated relaxivities (the relaxation rate enhancement brought by one millimol per liter of the gadolinium complex) vs. proton Larmor frequencies (proton NMRD profile) are shown on Figure 4.
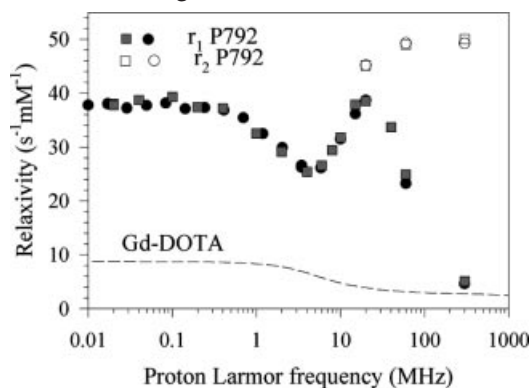

Figure 4. Proton NMRD data obtained at 310 K on a 0.285 mм solution (closed squares: r_1, open squares: r_2) and on a 1 mм solution (closed circles: r_1, open circles: r_2). The proton NMRD profile of DOTA-Gd (dashed line) has been added for comparison.

The longitudinal relaxivities show a hump at high magnetic fields in good agreement with the large size of the gadolinium complex and thus with its slow rotation. The values are remarkably larger, not only as compared to the parent complex DOTA-Gd, but also with respect to another large molecular weight derivative, the P760.[9] Longitudinal relaxivity reaches its maximum (38–40 s^{-1} mM^{-1}) be-

tween 15 and 40 MHz (0.35 and 0.94 T) while the transverse relaxivity levels off above 50 MHz (1.18 T).

In a first approach, ^1H NMRD curves were fitted according to the classical innersphere model described by Solomon[25] and Bloembergen[26] and to the outersphere model described by Freed.[27] In these fittings, the number of water molecules coordinated to the Gd^{3+} ion was fixed to 1 in agreement with the luminescence data. Some other parameters were also fixed: r, the distance between the proton nuclei of the innersphere water molecule and the Gd^{3+} (0.31 nm); D, the relative diffusion constant (3×10^{-9} $m^2 s^{-1}$); and d, the distance of closest approach for the outersphere contribution (0.36 nm[28,29]). τ_V and τ_{SO} (the electronic relaxation time at zero field), describing the elec-
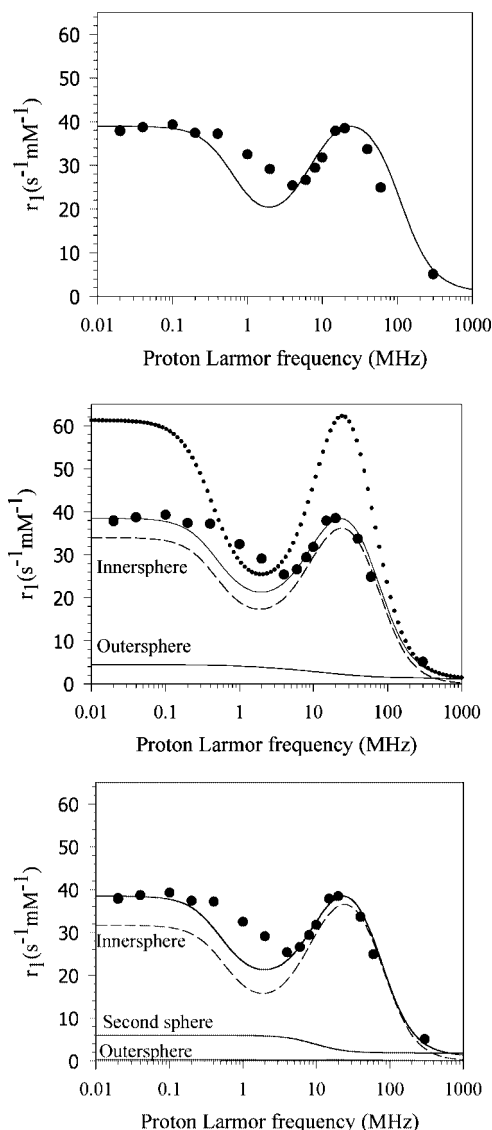

Figure 5. Theoretical fittings of the proton NMRD data obtained at 310 K using outersphere and innersphere models with τ_M fixed to 96 ns or τ_M adjusted (top and middle graphs; respectively) and using outersphere and innersphere models with second sphere water molecules included (bottom graph). The dotted line in the middle graph corresponds to the theoretical optimal relaxivity calculated with τ_M optimum ($\tau_M = 20$–30 ns).

tronic relaxation times, were optimized for the outersphere and the innersphere contributions simultaneously. τ_R was allowed to fluctuate around the values obtained by ^{17}O NMR and τ_M was fixed to 96 ns. A τ_R^{310} value of 1.7 ns and a τ_{SO} value of 538 ps were obtained but in the high field region, the fitted values are larger than the experimental ones (Figure 5 and Table 2). A second adjustment was therefore performed in which τ_M was adjusted. A satisfactory fit of the data could be obtained at low fields and at high fields with a τ_R^{310} value of 3.2 ns and a τ_M value of 240 ns (Figure 5 and Table 2). These values are significantly larger than those determined by ^{17}O NMR (2.4 ns and 80–96 ns, respectively). The τ_{SO} value is also larger than that reported for the parent compound DOTA-Gd (τ_{SO}^{310} = 480 ps).[22] These discrepancies could be explained by the fact that the model used to fit the ^1H NMRD curve is not the most appropriate to describe the behavior of slowly tumbling paramagnetic molecules.[30] They also could result from the presence of a second sphere of water molecules due to the hydrophilic nature of the substituents of the macrocyclic core of P792.

Table 2. Parameters obtained from the fitting of the proton NMRD profile of P792 at 310 K.

	Theoretical model with innersphere and outersphere water molecules; τ_M set to 96 ns	Theoretical model with innersphere and outersphere water molecules; τ_M adjusted	Theoretical model with innersphere, outersphere and second sphere water molecules
d [nm]	0.36	0.36	0.45
D [10^{-9} m^2 s^{-1}]	3.0	3.0	3.0
r [nm]	0.31	0.31	0.31
τ_R [ns]	1.7	3.2	2.7
τ_M [ns]	96	240	200
τ_{SO} [ps]	538	730	520
τ_V [ps]	40	26	29
τ_{SS} [ps]			25
r_{ss} [nm]			0.40
q_{ss}			10

In a third approach, the interaction between the gadolinium ion and water molecules in its second sphere was therefore included in the theoretical model.[31] The hydrogen atoms of these water molecules were assumed to be at a distance of 0.4 nm and the distance of closest approach was set to 0.45 nm. The experimental data were fitted, as shown in Figure 5, with a τ_R^{310} value of 2.7 ns which is similar to the value obtained above by the analysis of the paramagnetic longitudinal rate of oxygen-17. The value found for τ_M (200 ns) is closer to the value obtained by oxygen-17 NMR spectroscopy. The τ_{SO} (520 ps) and τ_V (29 ps) values are also in better agreement with those reported for the parent compound DOTA-Gd. In this fitting, 10 water molecules are found in the second sphere. Their interaction with the paramagnetic center is modulated by a correlation time called τ_{SS} which depends on the rotational correlation time and on the residence time of the second sphere water molecules. A value of 25 ps was found. However, it should be

pointed out that both last fittings give similar results and that the choice of the "best" theoretical model is quite subjective.

Nevertheless, it emerges from these data that the rotational correlation time is approximately equal to 2–3 ns at 310 K and that the value of the electronic relaxation rate at low fields is similar to that of the parent complex DOTA-Gd.

Finally, it can be calculated that the observed relaxivity is not optimal and that values of the order of 60 s^{-1}mm^{-1} between 20–60 MHz could be obtained if the water residence time was of the order of 20–30 ns (Figure 5).

Interaction of P792 with Blood Proteins

When small gadolinium complexes bind non-covalently to HSA, an increase of their paramagnetic relaxation rate is usually observed at magnetic fields ranging from 10 to 60 MHz due to the slowing down of the rotational motion of the bound complex. Proton relaxation rate measurements were thus performed at 20 and 60 MHz and 310 K on P792 (0.46 mm) solutions in human serum (Table 3). The increase of the longitudinal and transverse apparent relaxivities is minimal and can be explained by the slight increase in viscosity of the medium as compared to water solution. Alternatively, these data show either the absence of interaction between P792 and blood proteins, or that there is an interaction but that the rotational correlation time of the paramagnetic center is not significantly modified by the binding.

Table 3. Apparent relaxivities in water and human serum solutions of 0.46 mm of P792 at 310 K.

	20 MHz	60 MHz
r_1 [s^{-1}mm^{-1}] (water)	42±1.5	26.7±0.6
$r_1$app [s^{-1}mm^{-1}] (serum)	47.7±1.0	26.9±0.5
r_2 [s^{-1}mm^{-1}] (water)	50±1.4	53.3±2.9
$r_2$app [s^{-1}mm^{-1}] (serum)	59.4±1.3	65.1±1.5

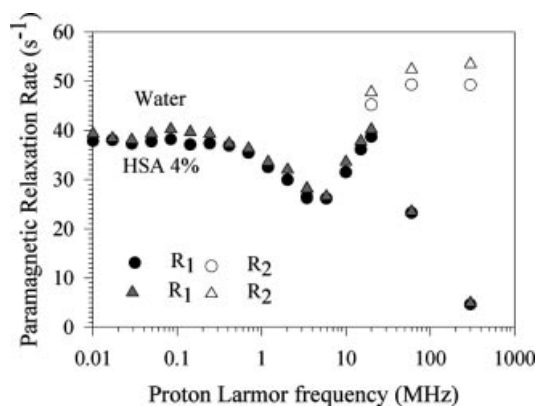The influence of HSA 4% on the longitudinal and transverse NMRD profiles was also investigated (Figure 6). The

Figure 6. Proton NMRD data obtained at 310 K on a 1 mm solution in water (closed circles: R_1p, open circles: R_2p) and HSA 4% (closed triangles: R_1p, open triangles: R_2p).

similarity of the paramagnetic R_1 data over the whole range of magnetic fields and of the paramagnetic R_2 data at high fields confirms that P792 does not bind to serum albumin.

The absence of a significant interaction with blood proteins must be related to the observed relatively fast clearance of P792 from mice plasma ($t_{1/2}\beta$ = 22 min)[6] or from rabbit plasma ($t_{1/2}\beta$ = 41 min)[8] as compared to the much larger values reported for MRI contrast agents like MS-325 ($t_{1/2}\beta$ = 155 min in rabbits)[32] and MP2269 ($t_{1/2}\beta$ = 142 min in rabbits);[33] known for their interaction with HSA.

Conclusions

P792, a new tetra-substituted macromolecular DOTA-Gd derivative, is a very interesting MRI contrast agent due to its hydrophilic properties, its very high longitudinal and transverse relaxivities in the imaging magnetic fields (> 30 $s^{-1}mM^{-1}$), and its very high stability with respect to Zn^{II} transmetallation. This complex, which does not bind significantly to serum proteins, is consequently cleared very rapidly by the kidneys. On the other hand, its in vivo relaxivity is independent of concentration; unlike what is observed for contrast agents binding to serum albumin.[34,35]

Experimental Section

Chemicals: All commercial chemicals [$ZnCl_2$ (Merck) and Titrisol buffer pH 7.0 (Merck)] were used without further purification. Non-defatted human albumin (fraction V) was purchased from Sigma (Bornem, Belgium). The synthesis of P792 has been described previously.[7]

Luminescence Studies on the Eu Complex: The Eu^{III} phosphorescence emission spectra were acquired with an LS-50B Perkin−Elmer spectrofluorimeter equipped with a pulsed Xenon lamp and a Hamamatsu R928 photomultiplier tube operating in time-resolved mode. 7×10^{-3} M and 5.5×10^{-3} M solutions of the Eu^{III} complex in H_2O and D_2O, respectively, were used in these experiments. Lifetimes (τ) were obtained by monitoring the emission intensity at 616 nm, resulting from pulsed excitation at 395 nm. Excitation and emission slit widths of 5 nm and 20 nm were used, respectively, and the gate time was 0.4 ms. 160 (H_2O) or 260 (D_2O) different delay times were used. The decays of the europium emitting state were fitted by an equation of the form $I(t) = I(0)\exp(-t/\tau)$ using a current software fitting procedure (Excel). High correlation coefficients were observed in each case (typically 0.999). Error limits on the lifetime are equal to 5%. The number of water molecules (q) bound to the inner coordination sphere of the europium ion was quantified by using either the original equation proposed by Horrocks and Sudnick[16] or the equation reported by Beeby et al.[17]

NMR Spectroscopy: Proton Nuclear Magnetic Relaxation Dispersion (NMRD) profiles extending from 0.24 mT to 1.2 T were recorded with Field Cycling Relaxometers (Field Cycling Systems, Oradell, New Jersey, USA and Stelar, Mede, Italy) on 0.6 mL solutions contained in 10-mm o.d. tubes. Proton relaxation rates were also measured at 0.47 T, 0.94 T, 1.5 T and 7.05 T with Minispec PC-120, PC-140, mq-60 and AMX-300 spectrometer. All these instruments were from Bruker (Karlsruhe, Germany). ^1H NMRD data were fitted according to the theoretical innersphere model de-

scribed by Solomon[25] and Bloembergen[26] and to the outersphere contribution described by Freed.[27] Calculations were performed with previously described software.[36,37]

Transmetallation by Zn^{II} ions was evaluated at 310 K by the decrease of the water proton longitudinal relaxation rate at 20 MHz (Bruker Minispec PC 120) of buffered solutions (pH 7, phosphate buffer: [KH_2PO_4] = 26 mM, [Na_2HPO_4] = 41 mM) containing the gadolinium complex and Zn^{II} according to a procedure previously described.[23]

^{17}O spectra were recorded on 2 mL samples (10-mm o.d. tubes) on a Bruker AMX-300 spectrometer (Bruker, Karlsruhe, Germany) using a broadband probe and a Bruker BVT-2000 unit for temperature control. ^{17}O transverse relaxation times of distilled water (pH = 6.5) were measured using a CPMG sequence and a subsequent two parameters fit of the data points. The 90° and 180° pulse lengths were respectively 25 µs and 50 µs. ^{17}O T_2 of water in P792 ([P792] = 7 mM, pH = 6.0–7.0) was obtained from linewidth measurement. All ^{17}O spectra were proton decoupled. The procedures for the analysis of the ^{17}O data have been described elsewhere.[20]

All T_1 measurements at 7.05 T (^1H, ^{17}O) were performed with an inversion recovery or fast inversion recovery Fourier transform technique and a subsequent three parameters fit of the peak heights.

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