Synthesis and Physicochemical Characterization of Gd-C_4-Thyroxin-DTPA, a Potential MRI Contrast Agent. Evaluation of Its Affinity for Human Serum Albumin by Proton Relaxometry, NMR Diffusometry, and Electrospray Mass Spectrometry

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Gd-C_4-thyroxin-DTPA, a potential MRI contrast agent, was synthesized from Gd-DTPA and thyroxine, which interacts strongly with human serum albumin (HSA). It was characterized in water by its relaxometric properties and its stability versus zinc transmetalation. The affinity of the complex for HSA was studied by using three different methods: proton relaxometry, NMR diffusometry, and electrospray mass spectrometry. From the results, it appears that Gd-C_4-thyroxin-DTPA exhibits a relatively high relaxivity ($r_1 = 9.01$ s^{-1} mM^{-1} at 1.5 T and 310 K), a good stability versus zinc transmetalation, and a strong interaction with HSA ($K_a \sim 10\,000$ M^{-1} with two binding sites). The kinetics of the exchange between the bound and the free form of the complex was evaluated by the NMR diffusometry technique. Competition experiments have allowed the assignment of the chelate's binding site on HSA.

1. Introduction

Contrast agents for magnetic resonance imaging (MRI) are mainly gadolinium complexes.[1−3] These paramagnetic chelates improve the MR image contrast by decreasing primarily the water proton longitudinal relaxation time in the regions of the body where they have accumulated. The efficacy of a gadolinium complex, determined by its longitudinal relaxivity (r_1), is thus defined as the increase of the water proton longitudinal relaxation rate induced by 1 mmol of gadolinium per liter of solvent.

At present, the contrast agents commercially available are characterized by a relatively low relaxivity ($r_1 \leq 5$ s^{-1} mM^{-1} at 1.5 T and 310 K). However, the relaxivity of a gadolinium contrast agent is governed by several structural and dynamical factors, so that efficient molecular contrast agents could theoretically be designed. Among these parameters, the rotational correlation time (τ_R) can be modulated by adjusting the size of the complex. Indeed, at the magnetic fields currently used in MRI (0.5−3.0 T), a slower rotational motion of the gadolinium complex, corresponding to a higher value of τ_R, would theoretically lead to a better relaxivity.[4] This can be achieved by increasing the molecular size of the paramagnetic complex[5,6] or by covalent or noncovalent binding to a macromolecule such as human serum albumin (HSA). This latter strategy was successfully achieved, for example, in the case of Gd-EOB-DTPA (Primovist, Bayer HealthCare), Gd-BOPTA (MultiHance, Bracco), or MS-325 (Vasovist/AngioMARK, Bayer HealthCare, Mallinckrodt).[7−12]

Human serum albumin is one of the most abundant proteins in the human body, with a concentration in the plasma of 4% or 0.6 mM. Among its numerous functions in the body, the transport of metabolites is one of the most important and, as a consequence, HSA is well-known for having the ability of binding a large variety of ligands. Several binding sites exist on HSA; however, according to the model established by Suddlow et al., two major binding sites, named Suddlow site I and Suddlow site II, bind small organic ligands.[13,14] Suddlow site I, located in the subdomain IIA, is known for binding very different ligands, such as salicylate, warfarin, or bilirubin. Suddlow site II, located in the subdomain IIIA, is a hydrophobic pocket which binds small aromatic carboxylic acids, such as L-tryptophan or ibuprofen.[15]

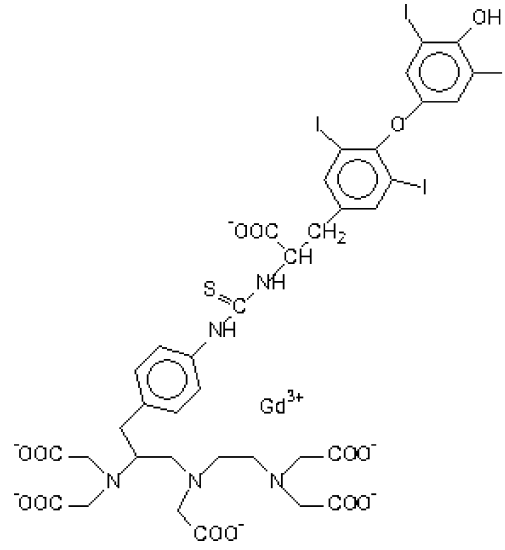

Figure 1. Chemical structure of Gd-C_4-thyroxin-DTPA.

In the present work, L-thyroxin, a thyroid hormone which is known to bind to thyroxin binding globulin (TBG) ($K_a = 9.08 \pm 0.62 \times 10^9$ M^{-1} with $n \sim 1$ at 310 K),[16] transthyretin (TTR) ($K_a = 1.3 \times 10^7$ M^{-1} with $n \sim 1$ at 310 K),[17] and HSA ($K_a = 1.01 \times 10^6$ M^{-1} with $n = 1.9$ at 310 K),[18] was coupled to a derivative of Gd-DTPA carrying a *p*-isothiocyanatobenzyl substituent. The resulting complex, Gd-C_4-thyroxin-DTPA (Figure 1), was then characterized in aqueous solution and in the presence of human serum albumin. Indeed, despite the highest affinity of thyroxine for TBG and TTR, the study of

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the interaction of Gd-C_4-thyroxin-DTPA with HSA was preferred because HSA is by far the most abundant protein in the human plasma (TBG circulates at a concentration of only 0.27×10^{-6} M as compared to concentrations of 4.6×10^{-6} M for TTR and 640×10^{-6} M for HSA).[19] This last study was performed by using several techniques: proton relaxometry,[7−12,20−28] electrospray mass spectrometry,[20,29−31] and NMR diffusometry.[32−40]

The proton relaxometry technique is the most widely used for the evaluation of noncovalent interactions between a gadolinium complex and its target. Indeed, its principle rests on the difference between the relaxivity of the free paramagnetic chelate and that of the supramolecular assembly resulting from its binding to HSA. Classically, the procedure consists of measuring the water proton relaxation rate on solutions containing increasing concentrations of gadolinium complex and a concentration of HSA fixed to 4% or 0.6 mM. The titration curve is then fitted with eq 1 in order to estimate the association constant and the number of binding sites, assuming that all sites are identical and independent.

$$R_{1\mathrm{obs}}^{\mathrm{P}} = 1000\left\{r_1^{\mathrm{l}} l^{\mathrm{o}}(r_1^{\mathrm{C}} - r_1^{\mathrm{l}}) \left\{\frac{Np^{\mathrm{o}} + l^{\mathrm{o}} + K_{\mathrm{a}}^{-1} - \sqrt{(Np^{\mathrm{o}} + l^{\mathrm{o}} + K_{\mathrm{a}}^{-1})^2 - 4Nl^{\mathrm{o}}p^{\mathrm{o}}}}{2}\right\}\right\} \quad (1)$$

In this equation, l° and p° are the total concentrations of gadolinium complex and HSA, respectively, r_1^{l} and r_1^{c} are the relaxivities of the free and bound complex, respectively, K_{a} is the association constant, and N is the number of binding sites, assumed to be identical and independent.

The second method we used, NMR diffusometry, is based on the measurement by NMR of the molecular self-diffusion coefficient[41−43] of the ligand in the absence and in the presence of the macromolecule. In the case of a rapid exchange between the ligand and the macromolecule on the NMR time scales, the observed diffusion coefficient is given by eq 2:

$$D_{\mathrm{obs}} = x_{\mathrm{l}} D_{\mathrm{l}} + x_{\mathrm{c}} D_{\mathrm{c}} \quad (2)$$

where D_{l} and D_{c} are the diffusion coefficients of the free and bound ligand, respectively, and x_{l} and x_{c} are their respective molar fractions.

A titration experiment, where the diffusion coefficient is measured on several solutions containing increasing concentrations of the ligand and a constant concentration in HSA, allows thus to estimate the association constant and the number of binding sites, assuming that all sites are identical and independent (eq 3).

$$D_{\mathrm{obs}} = D_{\mathrm{l}} + (D_{\mathrm{c}} - D_{\mathrm{l}}) \left[\frac{Np^{\circ} + l^{\circ} + K_{\mathrm{a}}^{-1} - \sqrt{(Np^{\mathrm{o}} + l^{\mathrm{o}} + K_{\mathrm{a}}^{-1})^2 - 4Nl^{\circ}p^{\circ}}}{2l^{\circ}}\right] \quad (3)$$

Finally, mass spectrometry can be used to study noncovalent interactions through a soft ionization mode named electrospray (ESI-MS), which allows keeping intact noncovalent complexes between a macromolecule and a ligand. The advantage of this technique rests on the possibility to see directly the stoichiometry of the interaction from the mass spectrum of the macromolecule in the presence of the ligand. Indeed, each complex will produce a peak, which can be directly interpreted. Furthermore, the program MaxEnt1, which uses the maximum entropy method, allows reconstructing a singly charged mass spectrum from a multiply charged one. The integration of each peak then allows extracting the concentrations of each species, and the successive association constants can be calculated according to eqs 4 and 5:

$$\mathrm{P} + \mathrm{L} \overset{K_1}{\rightleftharpoons} \mathrm{PL}_1, \quad \text{where} \quad K_1 = \frac{[\mathrm{PL}_1]}{[\mathrm{P}][\mathrm{L}]} \quad (4)$$

$$\mathrm{PL}_1 + \mathrm{L} \overset{K_2}{\rightleftharpoons} \mathrm{PL}_2, \quad \text{where} \quad K_2 = \frac{[\mathrm{PL}_2]}{[\mathrm{PL}_1][\mathrm{L}]} \quad (5)$$

In these equations, $[PL_1]$ and $[PL_2]$ correspond to the concentrations of the successive complexes between HSA and the ligand, [P] is the concentration of the free protein, and [L] corresponds to the concentration of the free ligand.

2. Materials and Methods

2.1. Chemicals. 1-*p*-Isothiocyanatobenzyldiethylenetriaminepentaacetic acid was obtained from Macrocyclics (Dallas, TX), and thyroxine was purchased from Sigma (Bornem, Belgium). The ligand C_4-thyroxin-DTPA was synthesized using the synthetic procedure described for Gd-C_4-sulfaphenazol-DTPA[20] and was complexed with gadolinium for the measurements with the proton relaxometry and mass spectrometry techniques. For the NMR diffusometry experiments, the gadolinium ion was replaced by a poorly relaxing analogue because of the excessive broadening of the NMR signals induced by Gd^{3+} ions. The europium ion was selected because it has the advantage of shifting the NMR signals of the chelate away from those of HSA, making it easier to measure the diffusion coefficient. The use of a diamagnetic analogue, however, would have been also necessary in some experiments, but, in the present study, the lanthanum chelate could not be used because of its poor solubility. This set of diffusion experiments was thus carried out with the noncomplexed ligand. This choice rests on the assumption that the noncomplexed ligand has the same affinity as the complexed one since the part of the compound which is responsible for the binding to HSA is the "thyroxine part", which is grafted on the skeleton of DTPA. This "thyroxine part" being relatively far from the complexed ion, we can reasonably suppose that the noncomplexed ligand has the same affinity as the complexed one.

C_4-thyroxin-DTPA: ^{1}H NMR (D_2O; δ (ppm); 11.75 T; s, singlet; d, doublet; dd, double doublet; t, triplet): 2.65 (dd, 1H diastereotopic from a CH_2); 2.75 (dd, 1H diastereotopic from the CH_2 of thyroxin); 2.85 (s, 2H, 1 × CH_2); 2.9 (dd, 1H diastereotopic from a CH_2); 3 (s, 2H, 1 × CH_2); 3.05 (t, 1H, 1xCH); 3.15 (dd, 1H diastereotopic from the CH_2 of thyroxin); 3.25 (dd, 1H diastereotopic from a CH_2); 3.3 (dd, 1H diastereotopic from a CH_2); 3.8 (s, 10H, 5 × CH_2−COOH); 4.3 (t, 1H, 1 × CH from thyroxin); 7.08 (d, 2H, 2 × CH); 7.12 (d, 2H, 2 × CH); 7.2 (s, 2H, 2 × CH); 7.8 (s, 2H, 2 × CH). ES-MS $(MH + Na)^+$: 1340.

Gd-C_4-thyroxin-DTPA: ES-MS $(M + 4Na)^+$: 1560.

Eu-C_4-thyroxin-DTPA: ES-MS $(M + 4Na)^+$: 1554, 1556 (Eu isotopes); $(M + 5Na)^+$: 1576, 1578.

Human serum albumin (HSA, product no. A-1653, powder 96−99%) was purchased from Sigma (Bornem, Belgium) and was used without further purification for the relaxometric and NMR diffusion measurements. For the mass spectrometry experiments, HSA was desalted by five dilution-concentration steps using Microcon YM-10 from Millipore (Brussels, Belgium). The protein concentration was measured spectrophotometrically (UV: $\lambda = 280$ nm) on an 8452A diode array spectrophotometer (Hewlett-Packard, Brussels, Belgium). The HSA concentration was 0.6 mM (4%) for the proton relaxation rate analysis, 0.6 or 0.15 mM for the diffusometry technique, and 5 μM for the mass spectrometry experiments.

The relaxometric measurements were performed in water, whereas the diffusion measurements were carried out in a phosphate buffer (0.2 M NaH_2PO_4 + 0.2 M Na_2HPO_4, pH = 7.4) prepared with heavy water for the europium complexes and only in heavy water for the noncomplexed ligand because of its poor solubility in the phosphate buffer. The mass spectrometry measurements were performed in ammonium acetate, which only slightly disturbs the conformation of HSA.[29]

Additional competition experiments with salicylic acid (Acros Organics, Geel, Belgium) and ibuprofen (Sigma, Bornem, Belgium) were performed in order to identify the chelate binding site on HSA. These experiments were performed by proton relaxometry and NMR diffusometry. The Gd-chelate solubilized in the phosphate buffer (0.2 M NaH_2PO_4 + 0.2 M Na_2HPO_4, pH = 7.4) was used for the proton relaxometry technique, whereas the experiments by NMR diffusometry were performed with the Eu-chelate in the same buffer prepared with heavy water.

2.2. Proton Relaxation Rate Analysis. Longitudinal proton relaxation rates were measured at 0.47 T and 310 K on a Minispec mq-20 (Bruker, Karlsruhe, Germany) instrument with a standard inversion−recovery sequence.

Proton nuclear magnetic relaxation dispersion (NMRD) profiles were recorded on a Stelar relaxometer (Mede, Italy) working between 0.24 mT and 0.24 T. The additional relaxation rates at 0.47, 1.41, 7.05, and 11.75 T were measured on mq-20 and mq-60 Minispec systems, and on AMX300 and AVANCEII-500 spectrometers (all from Bruker, Karlsruhe, Germany).

The measurements of the transmetalation by Zn^{2+} ions were performed at 310 K and 0.47 T with a concentration of gadolinium complex and of Zn^{2+} ions of 2.5 mM, in a phosphate buffer ($[KH_2PO_4] = 0.026$ mol/L and $[Na_2HPO_4] = 0.041$ mol/L, pH = 7.0).[7,44]

2.3. NMR Diffusion Measurements. The measurements were performed on an Avance200 spectrometer and on an AVANCEII-500 spectrometer equipped with variable temperature high resolution diffusion probes (Bruker). Two sequences, the "PGSE" (pulsed gradient spin echo) and the "PGSTE" (pulsed gradient stimulated echo), were used.[41−43] For all measurements, the length of the pulsed gradient (δ) was equal to 1 ms, whereas the diffusion time (Δ) was equal to 4 ms with the PGSE sequence and to 100 ms with the PGSTE sequence. The gradient strength was calibrated with water ($D = 3 \times 10^{-9}$ m^2/s at 310 K), and the maximum gradient strength was 1200 G/cm. Depending on the concentration, the number of scans varied between 400 and 20 000. The temperature was maintained at 310 K by circulation of water in the gradient coil (water bath HAAKE UWK 45 for the spectrometer AVANCE200 and water bath BCU 20 for the spectrometer AVANCEII-500). The solutions of the Eu-chelate were prepared in a phosphate buffer made with D_2O, whereas the solutions of the noncomplexed ligand, C_4-thyroxin-DTPA, were prepared only in D_2O. The diffusion coefficients were obtained by a fit of the peak heights, manually measured, versus the field gradient using eq 6:

$$I = I_o \exp(-\gamma^2 g^2 D\delta^2(\Delta - \delta/3)) \quad (6)$$

where γ is the gyromagnetic ratio and g is the gradient strength.

A biexponential fit of the data, where one of the components was assumed to be governed by the diffusion coefficient of HSA, was necessary in order to calculate the diffusion coefficient of the noncomplexed ligand and of ibuprofen or salicylate (in the competition experiments) because of a signal overlap between the ligand and the HSA.

The free ligand diffusion coefficient (D_l) was measured separately on a solution of the free chelate. The obtained value was then corrected by a factor of 1.16 in order to take into account the viscosity increase due to the presence of HSA. The bound ligand diffusion coefficient (D_c) was considered to be equal to the diffusion coefficient of HSA and was measured on a solution of HSA ($D_b = 5.5 \times 10^{-11}$ m^2/s in the phosphate buffer and $D_b = 7 \times 10^{-11}$ m^2/s in water).

2.4. Electrospray Mass Spectrometry Measurements. Electrospray mass spectra were obtained on a Q-tof 2 (Micromass, Manchester, U.K.) instrument in the positive ion mode at a capillary voltage of 1.4 kV. Samples, dissolved in ammonium acetate (100 mM), were injected with needles at a flow rate of a few nL/min.[29] Each spectrum is the sum of approximately 400 scans. The raw spectra were then baseline-corrected before deconvolution, using the program MaxEnt1. All spectra were recorded at a cone voltage of 180 V and a source temperature of 353 K. The concentration of HSA was fixed at 5 μM in ammonium acetate, and the concentration of gadolinium chelate ranged from 5 to 20 μM.

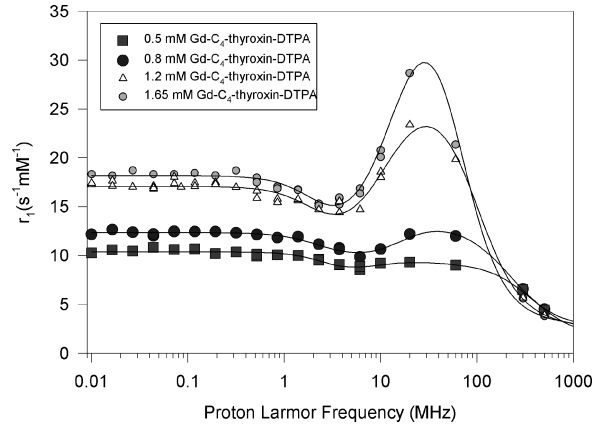

Figure 2. NMRD profiles of Gd-C_4-thyroxin-DTPA at different concentrations ($T = 310$ K). The lines through the data aim at guiding the eye.

3. Results and Discussion

3.1. Water Solution. ***3.1.1. Relaxometric Properties.*** The efficacy of a gadolinium chelate to enhance the image contrast is determined by its longitudinal relaxivity (r_1). The relaxivity of Gd-C_4-thyroxin-DTPA was therefore measured at different magnetic fields (NMRD profile) on solutions containing increasing concentrations of the chelate ($T = 310$ K) (Figure 2).

The observed relaxivity of our new complex increases with the concentration, particularly between 20 and 60 MHz, which is characteristic of an increasing rotational correlation time τ_R. This could be explained by the formation of aggregates between the molecules in solution. At first, it has been confirmed by the

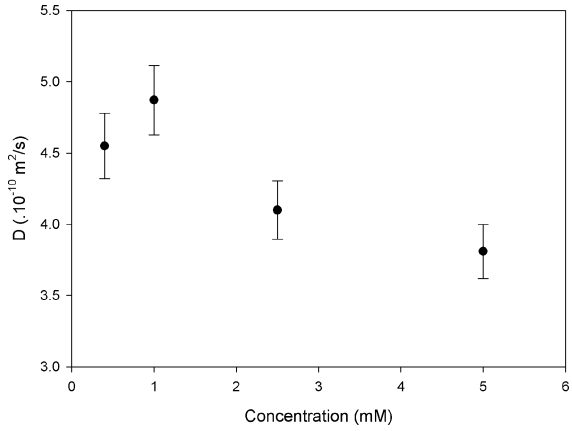

Figure 3. Evolution of the diffusion coefficient of C_4-thyroxin-DTPA with concentration ($B_0 = 11.75$ T, $T = 310$ K).

evolution of the relaxivity of the complex, at 20 and 60 MHz, as its concentration increases from 0.05 to 1.65 mM. The curves show no significant change of the relaxivity until a concentration of 0.65 mM ($r_1 \sim 9.02$ and 8.49 s^{-1} mM^{-1} at 20 and 60 MHz, respectively) and a marked increase when the concentration of the complex becomes higher (for a concentration of 1.65 mM of the chelate, $r_1 \sim 28.69$ and 21.36 s^{-1} mM^{-1} at 20 and 60 MHz, respectively). In addition, the aggregation of the gadolinium chelate has been confirmed by the NMR measurement of the diffusion coefficient (D) of the ligand C_4-thyroxin-DTPA. Indeed, as shown in Figure 3, D evolves from 3.81×10^{-10} m^2/s at 5 mM to 4.6×10^{-10} m^2/s at 0.4 mM, reflecting an increase of the hydrodynamic radius as the concentration increases. This supports an autoassociation probably resulting from intermolecular interactions between the aromatic parts of the molecules (π-stacking).

According to those relaxometric and diffusion measurements, the molecules of Gd-C_4-thyroxin-DTPA do not seem to be extensively aggregated at a concentration around 0.5 mM. An analysis of the NMRD profile of 0.5 mM Gd-C_4-thyroxin-DTPA solution was thus attempted in order to extract the parameters that are responsible for the relaxivity of the chelate. These are described by the models of Solomon,[45] Bloembergen,[46] and Freed,[47] which take into account two contributions: the inner-sphere contribution, which reflects the short distance interactions, and the outersphere contribution, which refers to the larger distance interactions. These models use different parameters that depend on the molecular structure and the dynamics of the gadolinium chelate. Some of them are quite similar for the low molecular weight gadolinium complexes, such as the distance between the protons of the coordinated water molecules and the gadolinium ion ($r = 0.30-0.31$ nm), the distance of closest approach for the water molecules ($d = 0.36$ nm), and the relative diffusion coefficient, which is close to that of pure water ($D = 3 \times 10^{-9}$ m^2/s at 310 K). The other parameters, that is, the number of coordinated water molecules (q), the residence time of these water molecules (τ_M), the rotational correlation time of the chelate (τ_R), the electronic relaxation time at very low fields (τ_{SO}), and the correlation time describing the modulation of the zero field splitting (ZFS) (τ_v), depend on the structure of the gadolinium complex.

Since the structure of our chelate is similar to that of the parent compound Gd-DTPA (Magnevist) and to that of other clinically used contrast agents, Gd-EOB-DTPA (Primovist)[9] and MS-325 (Vasovist),[10] for which the number of coordinated water molecules (q) is equal to one, we have considered this value of q for our chelate.

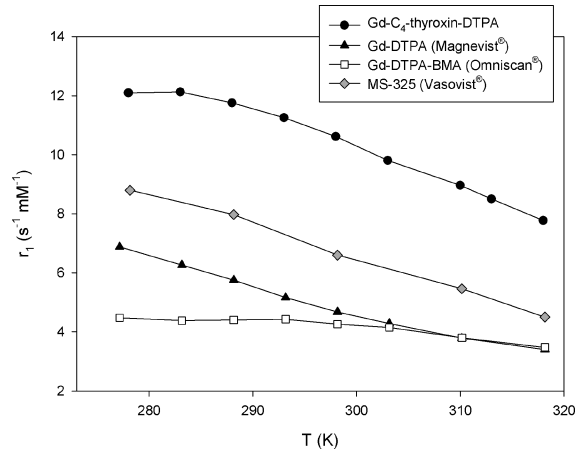

Figure 4. Evolution of the Gd-C_4-thyroxin-DTPA proton relaxivity with temperature ($B_0 = 0.47$ T). The curves previously obtained for Gd-DTPA (Magnevist, Bayer HealthCare) ($\tau_M = 143$ ns), MS-325 (Vasovist/AngioMARK, Bayer HealthCare, Mallinckrodt) ($\tau_M = 83$ ns), and Gd-DTPA-BMA (Omniscan, GE Healthcare) ($\tau_M = 967-1025$ ns) are added for comparison.[7]

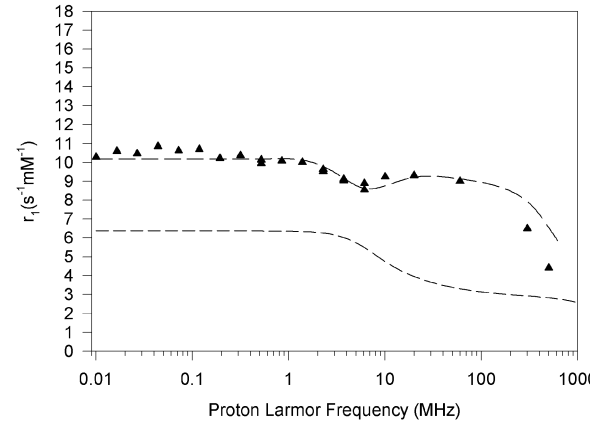

Figure 5. Fitting of the NMRD profile of 0.5 mM Gd-C_4-thyroxin-DTPA ($T = 310$ K). The curve for Gd-DTPA (dotted line) is added for comparison.

The residence time of this coordinated water molecule (τ_M) has been estimated by measuring the evolution of the Gd-C_4-thyroxin-DTPA relaxivity with temperature. Indeed, the continuous decrease of the relaxivity observed between 278 K ($r_1 = 12.10$ s^{-1} mM^{-1}) and 318 K ($r_1 = 7.77$ s^{-1} mM^{-1}) is typical of a fast exchange of the coordinated water molecule with the bulk water ($\tau_M \sim 100$ ns) (Figure 4). τ_M has thus a minute influence on the Gd-C_4-thyroxin-DTPA relaxivity at 310 K.[6]

The fitting of the NMRD profile of 0.5 mM Gd-C_4-thyroxin-DTPA with the theoretical models has allowed to adjust the rotational correlation time (τ_R), the electronic relaxation time at very low field (τ_{so}), and the correlation time for the modulation of the zero field splitting (τ_v) (Figure 5 and Table 1). The higher relaxivity of Gd-C_4-thyroxin-DTPA, as compared to Gd-DTPA, can be mainly explained by an increase of the rotational correlation time (τ_R) of the gadolinium chelate. The low quality of the fitting at high magnetic fields ($B_0 > 3$ T) could be explained by a low aggregation of the Gd complex leading to a "mixture" of rotational correlation times.

3.1.2. Complex Stability. The gadolinium ion is very toxic, and it is thus important to verify the kinetic inertia of the chelate toward the exchange with endogenous ions. The stability of the complex was tested in the presence of the zinc ion. In the blood, this ion is the most likely to challenge the gadolinium ion

TABLE 1: Results of the Fitting of the NMRD Profile of 0.5 mM Gd-C_4-Thyroxin-DTPA[a]

chelate	τ_R (ps)	τ_{so} (ps)	τ_v (ps)
Gd-C_4-thyroxin-DTPA	208	83	36
Gd-DTPA	59	82	23

[a] The other parameters which influence the relaxivity of the gadolinium chelate are fixed: $r = 0.3$ nm for Gd-C_4-thyroxin-DTPA and 0.31 nm for Gd-DTPA, $d = 0.36$ nm, $D = 3 \times 10^{-9}$ m^2/s, $q = 1$, and $\tau_M = 100$ ns. The data concerning Gd-DTPA are added for comparison ($\tau_M = 143$ ns). Previous works have shown that C-substituted derivatives of Gd-DTPA are characterized by a shorter distance between the protons of the coordinated water molecule and the gadolinium ion ($r = 0.3$ nm).[9,10]

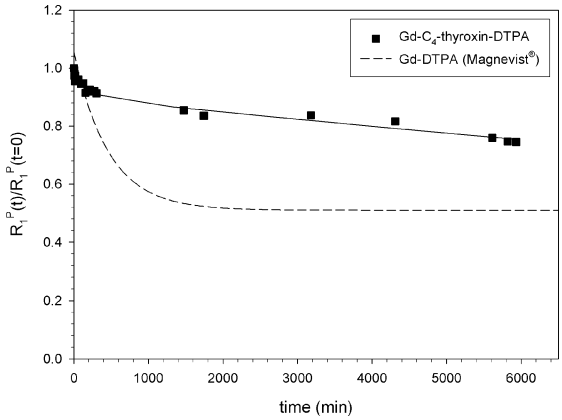

Figure 6. Evolution of the paramagnetic water proton relaxation rate of Gd-C_4-thyroxin-DTPA (2.5 mM) during the transmetalation process with the zinc ions (2.5 mM) in phosphate buffer (pH = 7.0) ($B_0 = 0.47$ T, $T = 310$ K). The previously obtained curve for Gd-DTPA is added for comparison (dashed curve).

because of a similar ionic radius and a quite large concentration. This experiment was performed in the presence of phosphate ions, which form an insoluble salt with the gadolinium ions. During the transmetalation process with the zinc ions, the released gadolinium ions react with the phosphate ions and precipitate. As a consequence, if transmetalation occurs, the water proton relaxation rate decreases with time. In the case of Gd-C_4-thyroxin-DTPA, a decrease of less than 30% is observed after 100 h, attesting to a very good stability of the complex. As shown by Figure 6, it is markedly more stable than the parent compound Gd-DTPA. This result confirms the tendency previously observed for the C-substituted derivatives of Gd-DTPA, which are very stable toward transmetalation with the zinc ion.[7,9,10]

3.2. HSA Solution. ***3.2.1. Proton Relaxometry.*** The NMRD profile of a solution of 0.5 mM Gd-C_4-thyroxin-DTPA and of 4% HSA was recorded at 310 K and compared to the NMRD profile of a solution of 0.5 mM Gd-C_4-thyroxin-DTPA in water (Figure 7). An important increase of the water proton paramagnetic relaxation rate is observed in the presence of HSA, particularly between 10 and 60 MHz, reflecting an increase of the rotational correlation time (τ_R), which is a consequence of the interaction of Gd-C_4-thyroxin-DTPA with HSA.

As the observed increase of the water proton paramagnetic relaxation rate depends on the proportion of the gadolinium chelate which is free and bound to the protein, it is possible to estimate the association constant and the number of binding sites by measuring the water proton relaxation rate on solutions of 4% HSA and of increasing concentrations of Gd-C_4-thyroxin-DTPA. This experiment is performed at 20 MHz, which is in the frequency range of the NMRD profile where the effect of

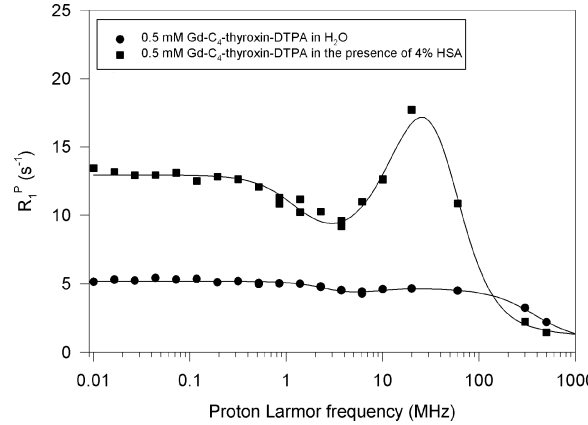

Figure 7. NMRD profiles of 0.5 mM Gd-C_4-thyroxin-DTPA in an aqueous solution and in the presence of 4% HSA ($T = 310$ K). The lines through the data aim at guiding the eye.

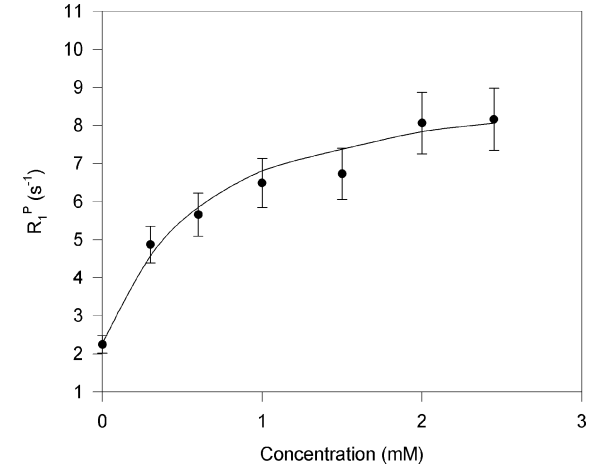

Figure 8. Evolution of the water proton relaxation rate measured on solutions containing various concentrations of HSA and a fixed concentration of Gd-C_4-thyroxin-DTPA (0.23 mM) ($B_0 = 0.47$ T, $T = 310$ K). The experimental error is estimated at 10%.

the interaction is maximal. The obtained curve is fitted with eq 1, where the relaxivities of the free gadolinium complex (r_1^l) and that of the bound one (r_1^c) have to be defined.

The relaxivity of the free gadolinium chelate (r_1^l) can be easily measured in a diluted aqueous solution, but that of the bound chelate (r_1^c) is more difficult to evaluate. An estimation can however be performed thanks to the inverse titration, where the concentration of Gd-C_4-thyroxin-DTPA is fixed at a relatively low value in order to favor the formation of the complex HSA/Gd-chelate and the concentration of HSA is increased. In this experiment, the water proton relaxation rate increases with the HSA concentration and reaches a maximum when all the binding sites of HSA are saturated. The value of the water proton relaxation rate at this maximum can be used in order to estimate the relaxivity of the bound chelate (r_1^c), and a value of 40.1 $\pm$ 5.3 s^{-1} mM^{-1} has been found for Gd-C_4-thyroxin-DTPA (Figure 8). It should, however, be noted that this value is only an estimate because as the concentration of HSA increases so does the viscosity of the solution, which is difficult to take into account in the fitting of the data.

The result of the titration experiment, where the concentration of HSA is fixed and that of Gd-C_4-thyroxin-DTPA varies, is shown in Figure 9. An association constant (K_a) of 10 100 $\pm$ 3930 M^{-1} with two binding sites was obtained by fitting the curve with eq 1. During this fitting, the relaxivity of the free

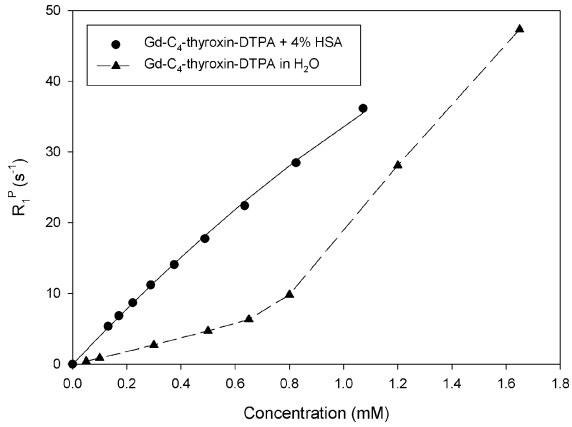

Figure 9. Evolution of the paramagnetic relaxation rate of water protons in the presence of various concentrations of the gadolinium chelate and of a 0.6 mM concentration of HSA ($T = 310$ K, $B_0 = 0.47$ T). The dashed curve represents the evolution of the water proton relaxation rate in the presence of various concentrations of Gd-C_4-thyroxin-DTPA in water.

gadolinium complex was allowed to vary by about 10% around the value measured in an aqueous solution for a low concentration of the chelate ($r_1^l = 7.5 \pm 0.4$ s^{-1} mM^{-1}), and the relaxivity of the bound chelates was allowed to vary by about 10% around the value obtained with the inverse titration ($r_1^c = 41.0 \pm 2.0$ s^{-1} mM^{-1}).

These results suffer from imprecision not only because of the hypothesis of identical and independent binding sites, but also and especially because of the parameter r_1^c which cannot be measured directly but only estimated. This parameter is indeed strongly correlated to the value of the association constant, and, as a consequence, a small variation of the relaxivity of the bound chelate can cause a significant variation of the association constant.

3.2.2. NMR Diffusometry. As an alternative to the proton relaxometry technique, we used the NMR diffusometry method. It also consists of performing titration experiments where the concentration of the chelate varies and the concentration of HSA is fixed, but the evaluation of the diffusion coefficients of the bound and the free ligand (D_c and D_l) can be performed more easily than the evaluation of the corresponding parameters (r_1^c and r_1^l) for the proton relaxometry technique. D_l can indeed be easily measured on an aqueous solution of the chelate, and D_c can be estimated by measuring the diffusion coefficient of the macromolecule.

The titration experiment was first performed on the europium chelate (see section 2.1), and no evolution of the diffusion coefficient was observed over the studied concentration range (Figure 10). Because of the large chemical shift range induced by the europium ion (of about 50 ppm), this can be explained by a slow exchange of the Eu-C_4-thyroxin-DTPA between its free and bound state on the NMR chemical shift time scale.[32]

A second titration experiment was performed on the non-complexed ligand C_4-thyroxin-DTPA in order to decrease the spectral width and hence to favor a fast exchange on the NMR chemical shift time scale (Figure 11). In this case, the presence of the HSA background in the spectra makes the measurement of the diffusion coefficient more laborious. The PGSTE sequence was thus used with a diffusion time Δ of 100 ms in order to partially suppress the signals of HSA. A biexponential fit of the diffusion curves was nevertheless necessary to take into account the small contribution of HSA to the ligand peaks.

The results show a clear evolution of the diffusion coefficient, which confirms the hypothesis of a slow exchange between the free and the bound ligand on the NMR chemical shift time scale in the case of the europium chelate. Moreover, the fitting of the data with eq 3 allows us to estimate an association constant

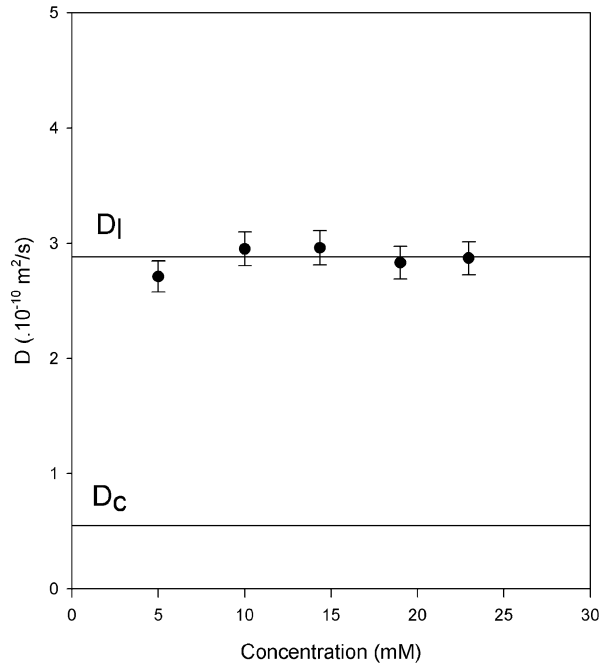

Figure 10. Titration experiment performed at 200 MHz with the PGSE sequence and a diffusion time Δ of 4 ms ($T = 310$ K). The concentration of HSA is 0.6 mM or 4%. D_l corresponds to the diffusion coefficient of the Eu-C_4-thyroxin-DTPA alone in solution corrected by a factor of 1.16 to take into account the higher viscosity of a 4% solution of HSA. D_c corresponds to the diffusion coefficient of HSA. An error of 5% is assumed for each measurement, which corresponds to the maximum statistical error obtained on the measured diffusion coefficients. All experiments were performed in deuterated phosphate buffer.

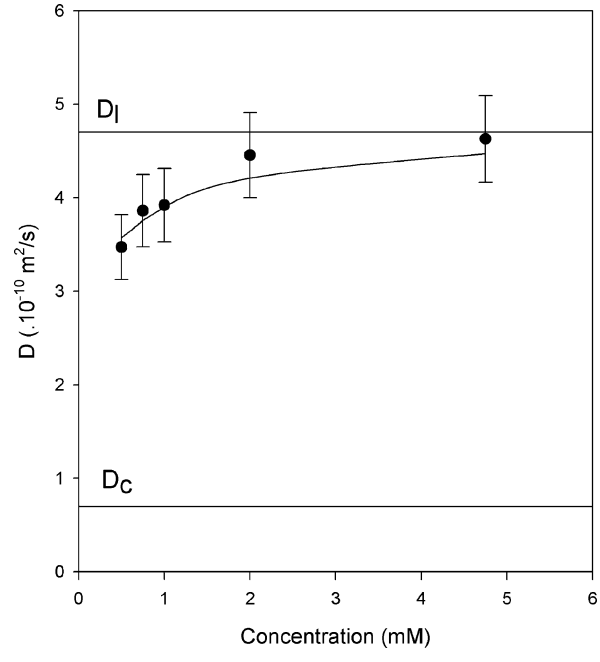

Figure 11. Titration experiment performed in D_2O at 500 MHz with the "pulsed gradient stimulated echo" sequence and a diffusion time Δ of 100 ms. An error of 10% is assumed for each measurement, which corresponds to the maximum statistical error obtained on the measured diffusion coefficients. D_l and D_c were measured as explained in Figure 10. The HSA concentration is equal to 0.15 mM because the low solubility of C_4-thyroxin-DTPA in water required a decrease of the HSA concentration.

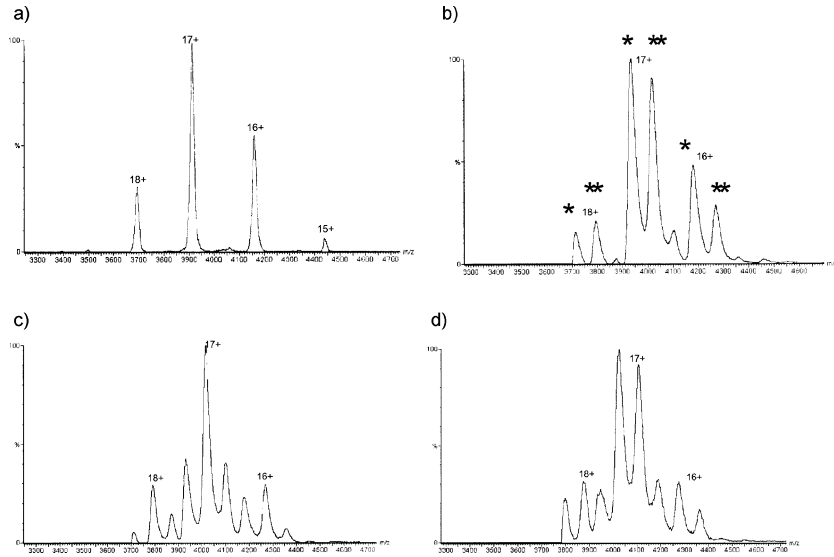

Figure 12. Mass spectra of 5 μM HSA alone (a) and in the presence of various concentrations of Gd-C_4-thyroxin-DTPA: 5 μM (b), 10 μM (c), and 20 μM (d). (*) Peaks of HSA and (**) peaks of the 1:1 complex HSA/Gd-chelate.

of 2500 $\pm$ 1400 M^{-1} with two binding sites, in moderate agreement with the results obtained by the proton relaxometry technique. It should, however, be noted that these values also suffer from imprecision because of the relatively important error associated with the measurement of the diffusion coefficient of the ligand C_4-thyroxin-DTPA in the presence of HSA. As previously indicated, some signals of HSA overlap those of the ligand, which makes it necessary to use a biexponential fitting of the diffusion data. Additionnally, the apparent discrepancy between the results obtained by proton relaxometry and NMR diffusometry could also be explained by the different concentrations used with the two techniques. Indeed, the diffusometry method does not allow to study solutions of a concentration below 0.5 mM of the noncomplexed ligand, because the overlap of the ligand peaks by those of HSA becomes too important. As a consequence, only solutions containing an excess of ligand were examined with the NMR diffusometry technique.

These two titration experiments performed on the europium chelate and on the noncomplexed ligand have the additional advantage of providing some information about the kinetics of the exchange. Indeed, with the europium chelate, the exchange is slow on the chemical shift time scale, so that the maximal chemical shift difference between the free and the bound species gives access to the minimal residence time of the ligand on HSA. As the NMR spectral width of the europium chelates is about 50 ppm, this value can be considered as the maximum chemical shift difference between the bound state and the free state. This corresponds to a frequency difference of about 10 000 Hz at 4.7 T. As the spectroscopic time scales can be defined by the relation $\kappa\tau \sim 1$ (where κ is a system property measured by spectroscopy such as a frequency or a rate, and τ is the characteristic time of the spectroscopic time scale), a characteristic time of 0.1 ms can be calculated. This value corresponds thus to the minimal residence time of the ligand on HSA. On the contrary, the exchange is rapid on the chemical shift time scale with the noncomplexed ligand, so that the minimal measurable difference between the bound and the free species gives now access to the maximal residence time of the ligand on HSA. That minimal chemical shift difference between the bound and free states can be evaluated to about 0.5 ppm, which corresponds, at 11.7 T, to about 250 Hz. The associated characteristic time is thus 4 ms. We can therefore estimate that the exchange time of this binding equilibrium ranges between 0.1 and 4 ms.[32]

3.2.3. Electrospray Mass Spectrometry. The mass spectra of 5 μM HSA in the presence of 5 and 10 μM Gd-C_4-thyroxin-DTPA clearly show, besides the spectrum of HSA, two signals which represent complexes corresponding to HSA bound to one and two gadolinium chelates, respectively (Figure 12b and c). When the concentration of the gadolinium chelate is brought to 4 times that of HSA, an additional signal appears while the HSA contribution vanishes (Figure 12d). This behavior is typical of a strong interaction between HSA and Gd-C_4-thyroxin-DTPA.

As explained above, the program MaxEnt1 allows to calculate the concentrations of the different adducts and therefore to extract the successive association constants. This treatment was applied to spectrum (c), for which the ratio between the concentration of HSA and Gd-C_4-thyroxin-DTPA is close to that used in the proton relaxometry and NMR diffusometry techniques. The obtained results show that the two first successive association constants are in the same range ($K_{a1} = 5 \times 10^5$ M^{-1} and $K_{a2} = 9 \times 10^4$ M^{-1}). This seems thus to confirm the stoichiometry obtained with the two previous techniques, where two sites were found on the assumption that they are identical and independent. On the basis of that result, it is possible to recalculate the association constant obtained by mass spectrometry through the model used with the two previous methods, that is, a single equilibrium with two equivalent binding sites. This calculation leads to an association constant of about 1×10^5 M^{-1}, which is close to the values obtained on the assumption of successive association constants.

This association constant is higher than that observed with the two previous methods, as it was already shown in previous studies.[20,28,29] This can be explained by the experimental conditions which are very different: measurements are performed in gas phase in mass spectrometry and in solution with proton relaxometry and NMR diffusometry. The strength of the interactions between the gadolinium chelate and HSA can thus be different. Moreover, the HSA is desalted by ultracentrifugation for the mass spectrometry measurements, whereas it is not for the measurements with the two other techniques. Finally, the temperature of the source is 353 K, which is higher than the temperature used in proton relaxometry and NMR diffusometry ($T = 310$ K).

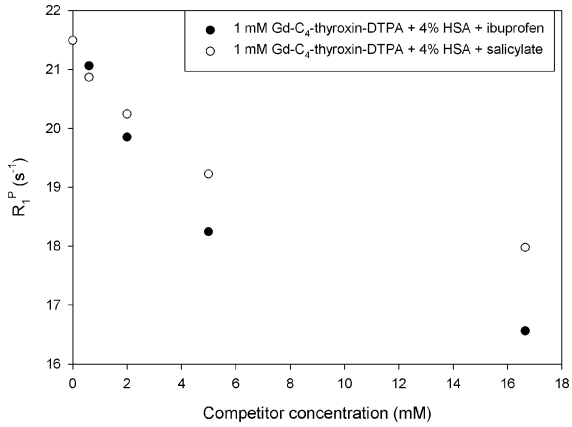

Figure 13. Water proton relaxation rate of a solution of 1 mM Gd-C_4-thyroxin-DTPA and 4% HSA in the presence of increasing concentrations of salicylate or ibuprofen ($B_0 = 0.47$ T, $T = 310$ K).

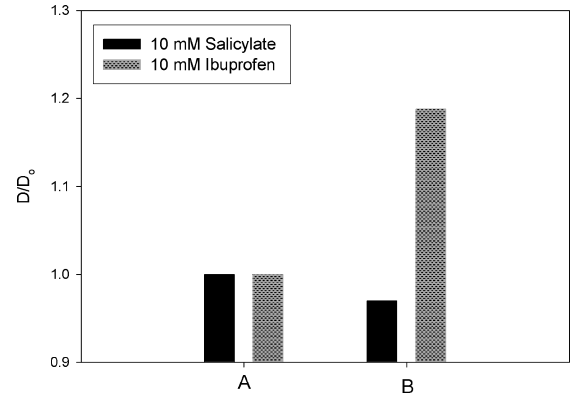

Figure 14. Competition experiments with ibuprofen and salicylate performed with the NMR diffusometry technique. (A) Diffusion coefficients of the competitors (D_0 ibuprofen $= 3.08 \times 10^{-10}$ m^2/s and D_0 salicylate $= 5.34 \times 10^{-10}$ m^2/s) in HSA solution, which were normalized to 1. (B) Relative diffusion coefficients of the competitors in the presence of HSA and of 2 mM Eu-C_4-thyroxin-DTPA (D/D_0) (D ibuprofen $= 3.66 \times 10^{-10}$ m^2/s and D salicylate $= 5.18 \times 10^{-10}$ m^2/s).

3.2.4. Competition Experiments. Competition experiments with molecules for which the binding site on HSA is known were performed in order to identify the binding site of Gd-C_4-thyroxin-DTPA. As previously mentioned, there are two main binding sites on HSA for the small organic molecules: Suddlow site I and Suddlow site II. Two molecules which bind to each of these sites were thus chosen for these competition experiments: salicylate ion, with its primary binding site on Suddlow site I and some secondary sites in Suddlow site II ($K_{a1} = 2.2 \times 10^5$ M^{-1}, $N = 1$; $K_{a2} = 1.6 \times 10^3$ M^{-1}, $N = 5$), and ibuprofen, with its primary binding site on Suddlow site II and some secondary sites in Suddlow site I ($K_{a1} = 2.7 \times 10^6$ M^{-1}, $N = 1$; $K_{a2} = 2.0 \times 10^4$ M^{-1}, $N = 6-7$).[15]

These experiments were first performed with the proton relaxometry technique by measuring the water proton relaxation rate of a solution of 1 mM Gd-C_4-thyroxin-DTPA and 4% HSA in the presence of increasing concentrations of ibuprofen or salicylate (Figure 13). The addition of a small concentration of both competitors causes similar and small decreases of the water proton relaxation rate, whereas, as the concentration of the competitors increases, ibuprofen induces the larger decrease of the water proton relaxation rate: the maximum drop is 30% with ibuprofen ([ibuprofen]/[Gd-C_4-thyroxin-DTPA] = 16.65), whereas salicylate induces a maximum decrease of 20% at the same concentration. In these concentration conditions, where a large excess of the competitor is added, the displacement of the gadolinium chelate is essentially induced at the secondary sites of the competitor, which could mean that Gd-C_4-thyroxin-DTPA binds preferably on Suddlow site I of HSA.

Competition was also assessed through the diffusometry technique performed on solutions containing 2 mM europium complex, 4% HSA, and 10 mM ibuprofen or salicylate. In these experiments, the diffusion coefficient of the competitor is measured and expected to increase if it is displaced from its binding sites by the europium chelate. These experiments are thus complementary to those performed with proton relaxometry, where the displacement of the chelate by the competitor was observed.

The results of these competition experiments show that the europium chelate induces an increase of the diffusion coefficient of ibuprofen (from 3.08×10^{-10} m^2/s in the absence of the complex to 3.66×10^{-10} m^2/s in its presence) but has no effect on the diffusion coefficient of salicylate (Figure 14). The Eu-chelate is thus unable to displace salicylate from its binding sites but competes with ibuprofen. Given the large excess of the competitor compared to the concentration of the Eu-Chelate and given that the association constant of the chelate with HSA, as obtained above, is much smaller than the association constant for the primary binding site of ibuprofen, it is likely that the Eu-chelate has displaced ibuprofen from its secondary sites. This seems thus to confirm that Gd- or Eu-C_4-thyroxin-DTPA binds on Suddlow site I of HSA, which is known to be able to bind several ligands at a time.[15]

4. Conclusion

Gd-C_4-thyroxin-DTPA, a new gadolinium complex, is characterized by a high water proton relaxivity and by a high stability versus zinc transmetalation. Moreover, the three techniques used to characterize its affinity for HSA, that is, proton relaxometry, NMR diffusometry, and electrospray mass spectrometry, show that the chelate interacts strongly with albumin.

Among these three techniques, proton relaxometry is the most appropriate method to characterize the affinity of a gadolinium complex for a macromolecule because it gives direct information about the efficacy of the gadolinium chelate as a MRI contrast agent in the presence of the macromolecule. However, this technique suffers from some imprecision due to the strong correlation between the estimated association constant and the relaxivity of the bound chelate (r_1^c), which can be only estimated. As an alternative, NMR diffusometry can be used because the diffusion coefficient of the bound chelate (D_c) can be more easily evaluated. Unfortunately, another source of imprecision comes from the difficulty to precisely measure the diffusion coefficient of the ligand C_4-thyroxin-DTPA because of the presence of the HSA signals in the spectra. Nevertheless, the quite good agreement between the proton relaxometry and NMR diffusometry data strengthens our confidence in the results.

Additional information about the kinetics of the exchange between the bound and the free state of the chelate has been obtained by the NMR diffusometry technique, while the electrospray mass spectrometry method has confirmed the stoichiometry of the interaction.

Finally, the competition experiments performed with salicylate and ibuprofen have allowed identification of the Suddlow site I as the major binding site of the chelate on HSA.

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