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ORIGINAL PAPER

Study of non-covalent interactions between MRI contrast agents and human serum albumin by NMR diffusometry

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Abstract The NMR diffusometry technique, based on the measurement of the diffusion coefficient of a ligand in the absence and in the presence of its macromolecular partner, was used to study the affinity for human serum albumin (HSA) of four gadolinium complexes, potential or already used magnetic resonance imaging contrast agents. Diamagnetic lanthanum(III) ion or europium(III) ion, which has the advantage of shifting the NMR signals far away from those of the macromolecule, was used to avoid the excessive broadening of the NMR signals induced by the gadolinium(III) ion. Titration experiments, in which the HSA concentration was kept constant and the concentration of the europium or lanthanum chelate was varied, were performed to evaluate the association constant and the number of binding sites. Some additional information about the kinetics of the exchange between the free and the bound chelate was also obtained. Competition experiments with ibuprofen and salicylate, which are ligands with a known affinity for the macromolecule and for which the binding site is known, were also performed to get information about the binding site of the contrast agents.

Keywords NMR · Contrast agents · Diffusion · Non-covalent interactions

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Introduction

Magnetic resonance imaging (MRI) contrast agents are nowadays routinely used to increase the water proton relaxation rate in the region where they accumulate and, thus, enhance image contrast [1–6]. Most of the commercial contrast agents clinically used are based on the gadolinium(III) ion and are characterized by a modest efficacy, a biodistribution limited to the vascular and extracellular spaces, and a rapid elimination by the kidneys. The challenge for chemists, thus, is to design more efficient and more specific contrast agents, in particular for molecular imaging, which is a new horizon for MRI. One way to increase the efficacy of a contrast agent is to slow down its rotational motion by a non-covalent interaction with an endogenous macromolecule, such as human serum albumin (HSA) [7–21].

HSA, a 60-kDa blood protein, is the most abundant protein in plasma, with a concentration of 4% (0.6 mM), and is known for binding a large variety of small ligands with a relatively high affinity. Two major binding sites can be distinguished, according to the Suddlow classification scheme [22–26]: Suddlow site I, located in subdomain IIA, which is reported in the literature as "a large and flexible region" [25], able to bind a wide diversity of ligands such as salicylate, warfarin, or bilirubin, and Suddlow site II, located in subdomain IIIA, which is able to bind small aromatic carboxylic acids, such as L-tryptophan, ibuprofen, or thyroxin.

To evaluate the non-covalent interaction of our contrast agents with HSA, we focused on NMR techniques. When compared with more traditional methods such as equilibrium dialysis or ultrafiltration, NMR has the advantage of not requiring a separation between the bound and the free ligand. Moreover, all molecules have active NMR nuclei;

therefore their derivatization, as needed for fluorescence spectroscopy, is not required. Several NMR methods allow the evaluation of non-covalent interactions [27–32]. The most widely used technique to study the binding between an MRI contrast agent and its target is proton relaxometry [7–21]. This method takes advantage of the relaxivity difference between the free and the bound contrast agent. However, the difficulty to evaluate the relaxivity of the bound contrast agent introduces an important imprecision on the association constant obtained.

In this work, we therefore focused on the observation of a hydrodynamic property of the ligand and more precisely the diffusion coefficient, which is an easy method to implement. Since the diffusion is a global molecular property, the diffusion coefficient of the ligand bound to the macromolecule can be evaluated by the measurement of the macromolecule diffusion coefficient, which represents a substantial benefit as compared with proton relaxometry.

The principle of the NMR diffusometry technique [33–50] for the study of non-covalent interactions rests on the difference between the diffusion coefficients of the small ligand, i.e. the contrast agent, free or bound to HSA. Indeed, if a non-covalent interaction takes place between both partners and if the exchange is fast with respect to the NMR timescales (chemical shift and diffusion timescales), the observed diffusion coefficient, i.e. the diffusion coefficient measured on one of the ligand resonances in the NMR spectrum, will be a weighted average between the diffusion coefficient of the free ligand and the diffusion coefficient of the bound ligand (Eq. 1):

$$D_{obs} = x_f D_f + x_b D_b \quad (1)$$

where D_{obs} is the observed diffusion coefficient, D_f and D_b are the diffusion coefficients of the free and bound ligands, respectively, and x_f and x_b are their molar fractions.

The association (characterized by K_a) between a protein P, with n identical and independent binding sites S, and a ligand L can be expressed by Eqs. 2 and 3:

$$L + S \rightleftharpoons LS \quad (2)$$

and

$$K_a = [LS]/[S][L] \quad (3)$$

where [LS], [L], and [S] are, respectively, the equilibrium concentrations of the bound ligands, of the free ligands, and of the free sites of the protein.

Assuming that the n binding sites are identical and independent, the following equations can be used:

$$nP_T = [LS] + [S] \quad (4)$$

and

$$L_T = [LS] + [L] \quad (5)$$

where P_T and L_T are the total concentrations of the protein and the ligand, respectively.

Equation 6 is obtained by combining Eqs. 3, 4, and 5:

$$[LS] = \frac{L_T + nP_T + 1/K_a - \left[(L_T + nP_T + 1/K_a)^2 - 4nP_T L_T\right]^{1/2}}{2} \quad (6)$$

Since $x_b = [LS]/L_T$, the combination of Eqs. 1 and 6 gives a relationship between D_{obs}, D_f, D_b, P_T, L_T, K_a, and n. Titration experiments, where the concentration of the ligand varies and the concentration of HSA is kept constant, thus allow us to estimate the values of the association constant and the number of binding sites.

It is, however, necessary to point out that if the exchange between the free and the bound ligands is slow on the chemical shift timescale and/or on the diffusion timescale, the resonances observed in the NMR spectrum will correspond to the signal of the free ligand, as the resonances corresponding to the bound ligand are too broad to be detected. Consequently, only D_f can be measured and no information about the binding of the ligand can be obtained. In this case, competition experiments with other ligands with medium affinity for the protein and sharing the same binding site are a good alternative [51–53]. This approach has, moreover, the advantage of providing some information about the binding site of the ligand. In the present study, we used two competitors: ibuprofen, which binds on Suddlow site II, with other secondary sites, and salicylate, which binds on Suddlow site I, with some secondary sites on Suddlow site II [22–25]. For these experiments, the diffusion coefficient of the competitor is measured and is expected to increase in the presence of the contrast agent if they share the same binding site.

Four complexes described in the literature were studied: Gd-DTPA, which does not interact with HSA [1, 2], and Gd-EOB-DTPA [7, 15], MP2269 [12–14], and Gd-C_4-sulfaphenazol-DTPA [8], all of which have a known affinity for HSA (Fig. 1).

Materials and methods

Chemicals

HSA (product no. A-1653, powder 96–99%) was purchased from Sigma (Bornem, Belgium) and used without further purification. Its concentration was 0.6 or 0.15 mM depending on the case considered.

To avoid the excessive broadening of the NMR signals due to gadolinium(III) ions, a poorly or non-relaxing analogue, such as europium(III) or lanthanum(III), was used. The paramagnetic europium(III) ion has the advantage of

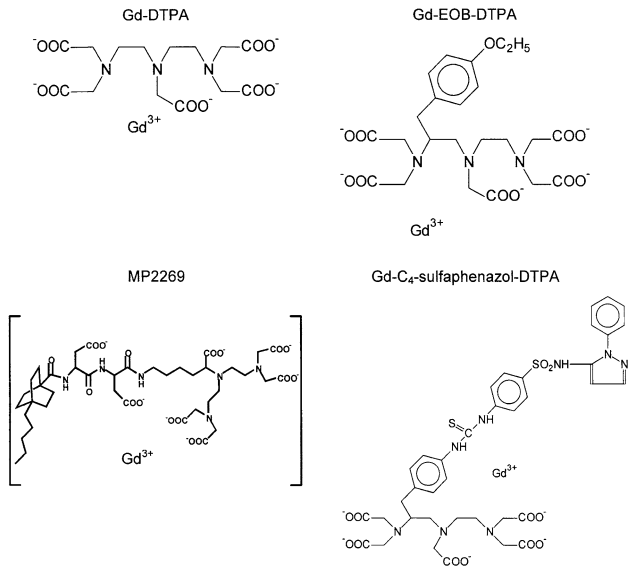

Fig. 1 Molecular structures of the four chelates studied

shifting the chelate NMR signals far away from those of HSA, facilitating the measurement of the diffusion coefficient. The use of the diamagnetic lanthanum(III) ion was, however, necessary in some cases.

DTPA was purchased from Fluka (Bornem, Belgium), EOB-DTPA was provided by Bayer Schering Pharma (Berlin, Germany), and the ligand MP-2269 was provided by Mallinckrodt (St. Louis, USA). The ligand C_4-sulfaphenazol-DTPA was synthesized as recently described by reaction between 1-*p*-isothiocyanatobenzyldiethylenetriaminepentaacetic acid (Macrocyclics, Dallas, USA) and sulfaphenazole (Sigma, Bornem, Belgium) [8]. All these ligands were complexed with europium(III) and/or lanthanum(III).

For the competition experiments, ibuprofen was purchased from Sigma (Bornem, Belgium) and salicylic acid was obtained from Acros Organics (Geel, Belgium).

The measurements were done in a phosphate buffer prepared in D_2O (0.2 M NaH_2PO_4 + 0.2 M Na_2HPO_4, pH 7.4), except for measurements with the lanthanum complexes, which were performed in pure D_2O because of their poor solubility in the buffer. Some measurements with europium chelates were also performed in D_2O.

NMR diffusion measurements

The NMR measurement of the diffusion coefficient was performed using two sequences: a pulsed gradient spin echo (PGSE) sequence and a pulsed gradient stimulated echo (PGSTE) sequence (Fig. 2) [54–56].

According to Eq. 7, the NMR signal decreases with gradient amplitude, allowing us to obtain the value of the diffusion coefficient:

$$I = I_0 \exp\left(-\gamma^2 g^2 D\delta^2(\Delta - \delta/3)\right) \tag{7}$$

where I is the amplitude of the NMR signal in the presence of gradient pulses, I_0 is the amplitude of the NMR signal without any gradient pulses, γ is the gyromagnetic ratio of the nucleus, g is the gradient amplitude, D is the diffusion coefficient, δ is the duration of the gradient pulses, and Δ is the diffusion time (i.e. the time between the two gradient pulses).

I_0 depends on the relaxation of the spins, and is different for the two sequences. In the PGSE sequence, only the transverse relaxation time T_2 induces the decrease of the signal, according to Eq. 8:

$$S(2\tau) = S_0 \exp\left(\frac{-2\tau}{T_2}\right) \exp\left(-\gamma^2 g^2 D\delta^2 \left(\Delta - \frac{\delta}{3}\right)\right) \tag{8}$$

where $S(2\tau)$ is the amplitude of the NMR signal, S_0 is the amplitude of the NMR signal without any gradient pulses and without any relaxation phenomenon, and τ is the half echo time.

In the PGSTE sequence, the spins are aligned along the z-axis during most of the experiment. The signal thus decreases owing to both the transverse and the longitudinal relaxations, i.e. T_2 and T_1 (Eq. 9):

$$S(2\tau + t) = \frac{S_0}{2} \exp\left(\frac{-2\tau}{T_2} - \frac{t}{T_1}\right) \exp\left(-\gamma^2 g^2 D\delta^2 \left(\Delta - \frac{\delta}{3}\right)\right) \tag{9}$$

For all measurements, δ is 1 ms, whereas Δ is 4 ms for the PGSE sequence and 100 or 150 ms for the PGSTE sequence.

The measurements were performed using an Avance 200 spectrometer (Bruker, Karlsruhe, Germany) or an Avance II 500 spectrometer (Bruker) equipped with a variable-temperature high-resolution commercial diffusion probe (Avance 200: DIFF/30 probe, 5 mm, maximum gradient 1,000 G cm^{-1}; Avance II 500: DIFF/30 probe, 5 mm, maximum gradient 1,500 G cm^{-1}). The sample volume (260 µL) was adjusted to match the height of the coil. The gradient strength was calibrated with water ($D = 3 \times 10^{-9}$ m^2 s^{-1} at 37 °C) and the temperature was maintained at 37 °C by a water flow in the gradient coil (HAAKE UWK 45 water bath for the Avance200 spectrometer and BCU 20 water bath for the AvanceII500 spectrometer).

To remove the HSA signals from the NMR spectra, a T_2 filter is usually applied (use of a Carr–Purcell–Meiboom–Gill PGSE or a Carr–Purcell–Meiboom–Gill PGSTE sequence). In our case, however, it was impossible to apply this procedure because the europium and lanthanum chelates have T_2 values on the same order as those of HSA. As explained above, this is not a problem for the europium chelates, because this ion has the advantage of shifting the

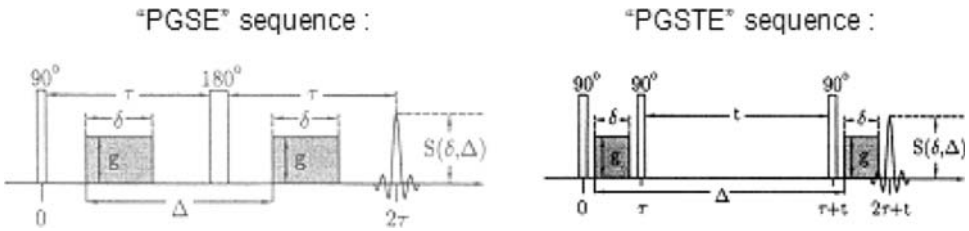

Fig. 2 NMR sequences used for the measurement of the diffusion coefficients [53]. *PGSE* pulsed gradient spin echo, *PGSTE* pulsed gradient stimulated echo

chelate's signals far away from those of HSA. For the lanthanum chelates, on the other hand, a biexponential fit of the diffusion curve, with one of the components fixed to the diffusion coefficient of HSA, was applied. The same method was used for the competition experiments.

For each titration experiment, the free contrast agent diffusion coefficient (D_f) was measured separately for a solution of the free contrast agent. The value obtained was then corrected by a factor of 1.16 to take into account the viscosity difference between a water solution of the contrast agent and the same solution in the presence of HSA. The bound contrast agent diffusion coefficient (D_b) was considered to be equal to the diffusion coefficient of HSA and was measured for a solution of HSA ($D_b = 5.5 \times 10^{-11}$ m^2 s^{-1} in the deuterated phosphate buffer and $D_b = 7 \times 10^{-11}$ m^2 s^{-1} in D_2O).

Results and discussion

Titration experiments

The titration experiments, where the diffusion coefficient (D) is measured as a function of the concentration of each europium chelate in HSA solution (4%), were first performed with the PGSE sequence and a diffusion time Δ of 4 ms (Fig. 3).

For Eu-DTPA, a very slight evolution of the diffusion coefficient is observed, which is coherent with the absence of affinity of this chelate for HSA. Gd-EOB-DTPA is known for having a medium affinity for HSA. This is confirmed by the curve obtained for Eu-EOB-DTPA shown in Fig. 3, which shows an evolution of the diffusion coefficient only for the lowest concentrations of the chelate. The theoretical fitting of the curve (see "Introduction") gave an association constant of 100 ± 218 M^{-1} with 1 ± 1.5 binding sites, in agreement with previous results obtained on Gd-EOB-DTPA in buffer solutions [57]. The affinity of the gadolinium chelate for HSA has been previously shown to be higher in the absence of salts [7, 15, 57]. This is confirmed by the measurements performed in D_2O for which a larger evolution of the diffusion coefficient of Eu-EOB-DTPA is observed and can be fitted with an association constant of $1.0 \times 10^4 \pm 4.7 \times 10^3$ M^{-1} with 1.2 ± 0.2 binding sites. For the two other chelates, Eu-MP2269 and Eu-C_4-sulfaphenazol-DTPA, no significant evolution of the diffusion coefficient could be observed, while the proton relaxometry technique has shown that their gadolinium analogues have moderate to high affinity for HSA [8, 12–14]. This apparent discrepancy can be explained by a slow exchange between the bound and the free form of the chelate on the NMR timescales, i.e. the chemical shift and/or the diffusion timescales. In fact, the literature describes the interaction of many ligands with HSA as a two-step phenomenon [25]. The first step is very fast (less than 1 ms) and corresponds to "a loose ionic attachment" of the ligand to the surface of the protein, while the second one is slower and corresponds to the "unfolding and reclosing of a hydrophobic pocket" in the protein where the ligand penetrates. This is thus an entropy-driven process because this "breathing" of the protein implies the penetration of water molecules during the unfolding process followed by the exclusion of these water molecules when the ligand binds and the pocket folds back. If the exchange between the bound and the free ligand is slow on the chemical shift timescale, peaks corresponding to the free state of the ligand and peaks corresponding to the bound state should be observed. However, given that the transverse relaxation times of the europium chelates are short at high magnetic fields ($T_2 \leq 10$ ms), the peaks are quite broad [58]. This makes it difficult to observe the peaks corresponding to the bound state because of their low intensity and their large linewidths due to Curie relaxation ($T_2 < 5$ ms) [59]. If the exchange between the bound and the free ligand is slow on the diffusion timescale (Δ), but rapid on the chemical shift timescale, we can expect to observe peaks at frequencies corresponding to the weighted averages between the bound and the free states and for which the diffusion curve can only be fitted with a biexponential curve, one component being the diffusion coefficient of the bound ligand and the other one being the diffusion coefficient of the free ligand. This treatment was applied to our data but did not give meaningful results, which seems to prove that the exchange is probably slow on the chemical shift timescale of the europium complexes.

Further measurements were thus performed on chelates of lanthanum, since this ion induces very small shifts in the NMR signals. A rapid exchange on the chemical shift timescale is thus expected, which allows us to perform the titration experiments. When lanthanum chelates are used,

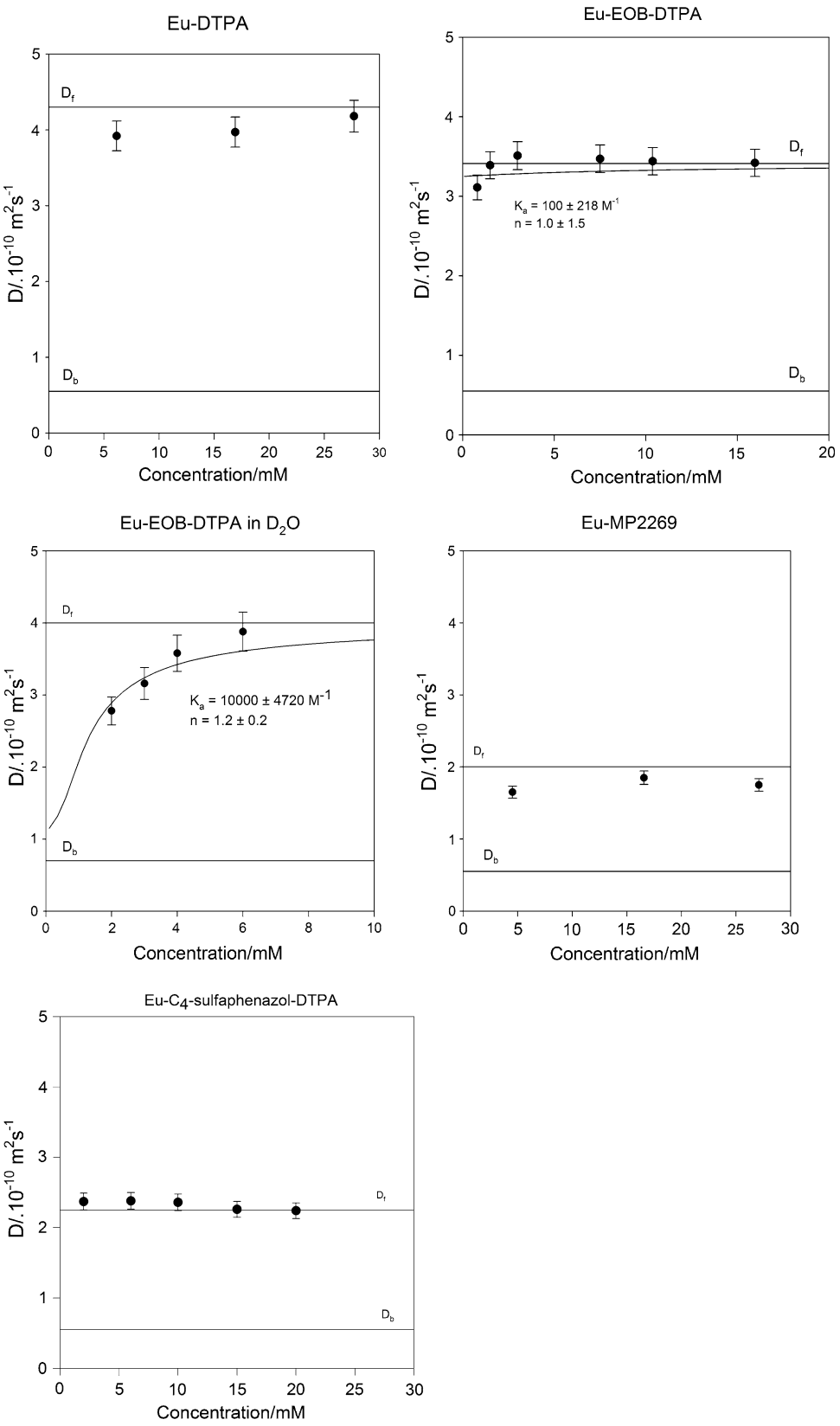

Fig. 3 Titration experiments for the europium chelates performed at 200 MHz for all compounds except for Eu-EOB-DTPA, which was studied at 500 MHz, with the "pulsed gradient spin echo" sequence and a diffusion time Δ of 4 ms. D_f corresponds to the diffusion coefficient of the europium chelate alone in solution corrected by a factor of 1.16 to take into account the higher viscosity of a 4% solution of human serum albumin (HSA). D_b 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 buffer except for experiments with Eu-EOB-DTPA, for which measurements were also carried out in pure D_2O

the HSA background in the NMR spectra makes it more difficult to measure the diffusion coefficient. The PGSTE sequence with a long diffusion time Δ of 100 or 150 ms was then used, to partially suppress the HSA signals. A biexponential fit of the diffusion curves was necessary, nevertheless, to take into account the small contribution of HSA to the ligand peaks (Fig. 4).

The measurements were first performed on La-DTPA to test that the use of a biexponential fit of the diffusion curve and of a longer diffusion time provide valuable results. It is reported in the literature that a long diffusion time Δ can cause a spectral editing of the fraction of the free ligand: its signal is preferentially attenuated because it diffuses faster than ligand molecules bound to the protein during the diffusion time. As a consequence, a decrease of the measured diffusion coefficient is induced [35]. As the data obtained for La-DTPA showed the same trend as those obtained for the europium chelate with the PGSE sequence and $\Delta = 4$ ms, we performed the titration experiments for the two other chelates with the same experimental conditions (Fig. 4). For La-MP2269 an enhancement of the diffusion coefficient of 60% is observed when the concentration increases from 2 to 16.5 mM. These data confirm our hypothesis of a slow exchange on the chemical shift timescale for the europium chelate analogue. The fitting of the curve gives an association constant of $5.3 \times 10^3 \pm 4.4 \times 10^3$ M^{-1} with 8.8 ± 1.1 binding sites, which corresponds to a global binding constant ($K_a n$) of about 4.7×10^4 M^{-1}, a value slightly larger than values reported in previous studies [12–14].

The titration experiment of La-C_4-sulfaphenazol-DTPA was performed with 0.15 mM HSA (i.e. 1% instead of 4%) because with an excess of ligand equivalent to 10 times the concentration of HSA the ligand signals "disappear" from the NMR spectrum, probably because they become too wide owing to the large fraction of ligand bound to HSA. Since the solubility of La-C_4-sulfaphenazol-DTPA in water does not allow us to work at higher concentrations of the

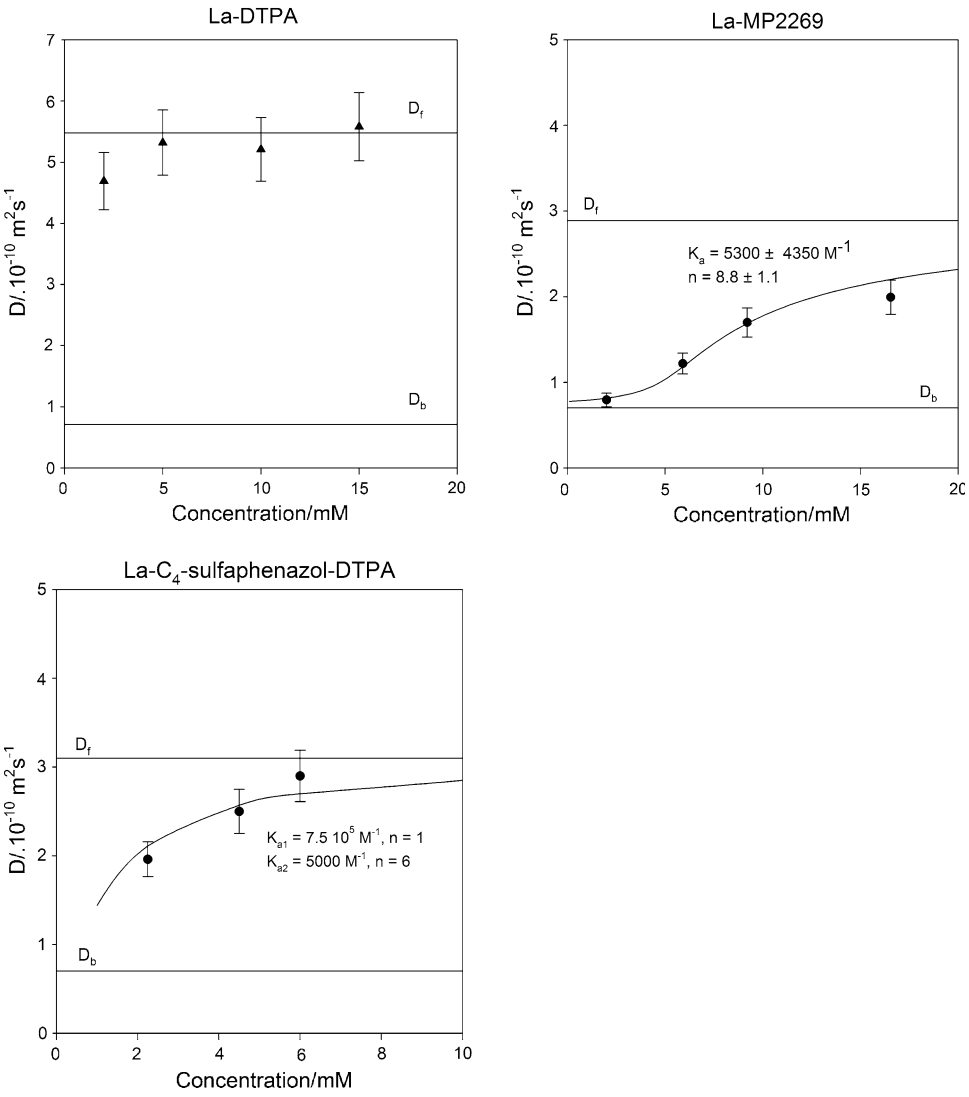

Fig. 4 Titration experiments performed in D_2O at 500 MHz, except for La-MP2269 (200 MHz), with the "pulsed gradient stimulated echo" sequence and a diffusion time Δ of 100 ms (La-C_4-sulfaphenazol-DTPA) or 150 ms (La-DTPA and La-MP2269). An error of 10% is assumed for each measurement, which corresponds to the maximum statistical error obtained on the measured diffusion coefficients. D_f and D_b were measured as explained in the legend to Fig. 3. The HSA concentration was 0.6 mM, except for La-C_4-sulfaphenazol-DTPA experiments, for which it was 0.15 mM (further explanations are provided in the text)

ligand, the HSA concentration was decreased. The experimental curve thus includes only three points, so an accurate fitting is not possible. Nevertheless, an analysis of the data was attempted taking into account the association of the gadolinum chelate analogue previously estimated by the proton relaxometry technique: a strong binding site with K_{a1} of 7.5×10^5 M^{-1} and two weaker binding sites with K_{a2} of 2×10^4 M^{-1} [8]. In the "Introduction" we mentioned that all sites were assumed to be identical and independent. If two types of sites (S_1 and S_2) exist, two equilibrium equations have to be considered:

$$S_1 + L_T \rightleftharpoons X \tag{10}$$

with

$$K_1 = \frac{x}{(L_T - x - y)(s_1 - x)} \tag{11}$$

and

$$S_2 + L_T \rightleftharpoons Y \tag{12}$$

with

$$K_2 = \frac{y}{(L_T - x - y)(s_2 - y)} \tag{13}$$

with s_1 and s_2 being the concentration of the two types of binding sites, K_1 and K_2 the association constants of these two binding sites, L_T the initial concentration of ligand, x the concentration of ligand bound to site s_1, and y the concentration of ligand bound to site s_2.

By combining Eqs. 11 and 13, we obtain Eq. 14:

$$y = \frac{K_2 s_2 x}{K_1 s_1 - x K_1 + x K_2} \tag{14}$$

Introducing this equation into Eq. 11 gives an equation of the third order (Eq. 15):

$$x^3(K_2 - K_1) + x^2\left(2K_1 s_1 - K_2 s_1 + K_2 s_2 + L_T K_1 - L_T K_2 + 1 - \frac{K_2}{K_1}\right) + x(-2L_T K_1 s_1 + L_T K_2 s_1 - K_1 s_1^2 - K_2 s_2 s_1 - s_1) + L_T K_1 s_1^2 = 0 \tag{15}$$

Equation 15 combined with Eq. 1 allows for the estimation of the diffusion coefficient.

Theoretical data calculated with a strong binding site with K_{a1} of 7.5×10^5 M^{-1} and six weaker sites with K_{a2} of 5,000 M^{-1}, which corresponds to the same global association constant (K_a n) for the weaker sites as obtained by the proton relaxometry technique [8], match the experimental data quite well (Fig. 4). It should be noted that these values must be considered as rough approximations since only three data points were available.

Nevertheless, these titration experiments thus provide an estimation of the association constant and of the number of binding sites in agreement with the results obtained with other techniques. They have the additional advantage of providing some information about the kinetics of the exchange. Indeed, with the europium chelates the exchange is slow on the chemical shift timescale. The NMR spectral width of the europium chelates is about 50 ppm and a maximum chemical shift difference of some tens of parts per million is expected 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 timescales 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 timescale), a characteristic time of 0.1 ms can be calculated. In contrast, the exchange is rapid on the chemical shift timescale with the lanthanum chelates, for which a minimum chemical shift difference between the bound and the free states of about 0.5 ppm can be expected. At 11.7 T, it corresponds to about 250 Hz. The associated characteristic time is thus 4 ms. We can therefore estimate that the exchange time between the bound and the free state ranges between these two limits.

Competition experiments

Interesting additional information can be provided by competition experiments with molecules having a moderate affinity for the macromolecule and for which the binding site is known. In these experiments, the diffusion coefficient of the competitor is measured and is expected to increase if the ligand interacts with the same binding site. Two competitors, ibuprofen and salicylate, were used in this study. Ibuprofen is a well-known anti-inflammatory drug which binds on Suddlow site II of HSA [$K_{a1} = 2.73 \times 10^6$ M^{-1} ($n = 1$), $K_{a2} = 1.95 \times 10^4$ M^{-1} ($n = 6$–7)], and salicylate is known for binding mainly to Suddlow site I of HSA [$K_{a1} = 2.2 \times 10^5$ M^{-1} ($n = 1$), $K_{a2} = 1.6 \times 10^3$ M^{-1} ($n = 5$)] [22]. Titration experiments were first performed for each of the competitors (Fig. 5) and reflect only the secondary binding sites of the two molecules, probably owing to a slow exchange on the main site. However, this is not a problem because if the chelates interact with the principal binding site of the competitor, the diffusion coefficient of the latter will be increased.

These competition experiments were performed on solutions containing 2 mM europium chelate, 4% HSA, and 10 mM ibuprofen or salicylate (Fig. 6). The advantage of using europium chelates in this study is that their NMR peaks are not superimposed on those of ibuprofen or salicylate.

The results clearly show no marked displacement of ibuprofen and salicylate in the presence of Eu-DTPA, which confirms that this chelate has no significant affinity

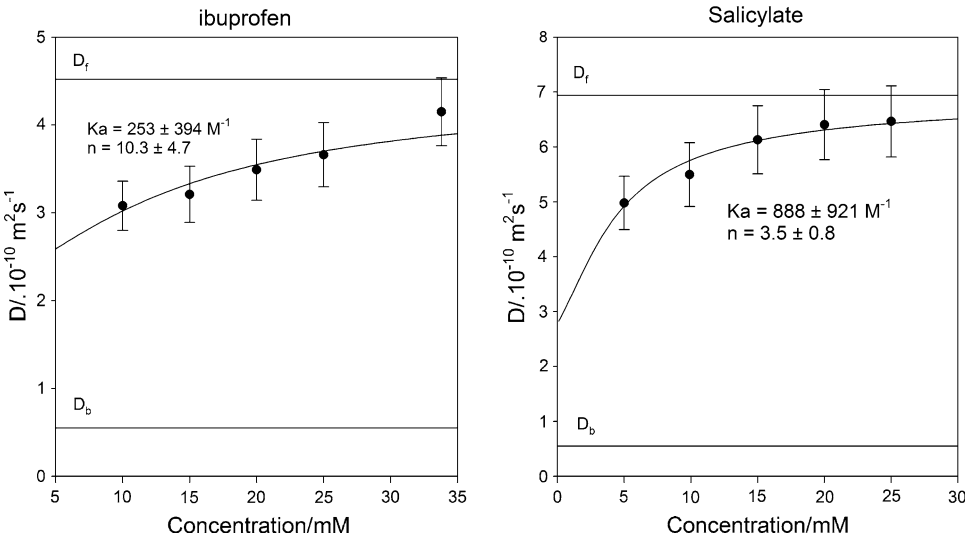

Fig. 5 Titration experiments performed in deuterated phosphate buffer at 200 MHz, with the "pulsed gradient spin echo" sequence and a diffusion time Δ of 4 ms. An error of 10% is assumed for each measurement, which corresponds to the maximum statistical error obtained on the measured diffusion coefficients

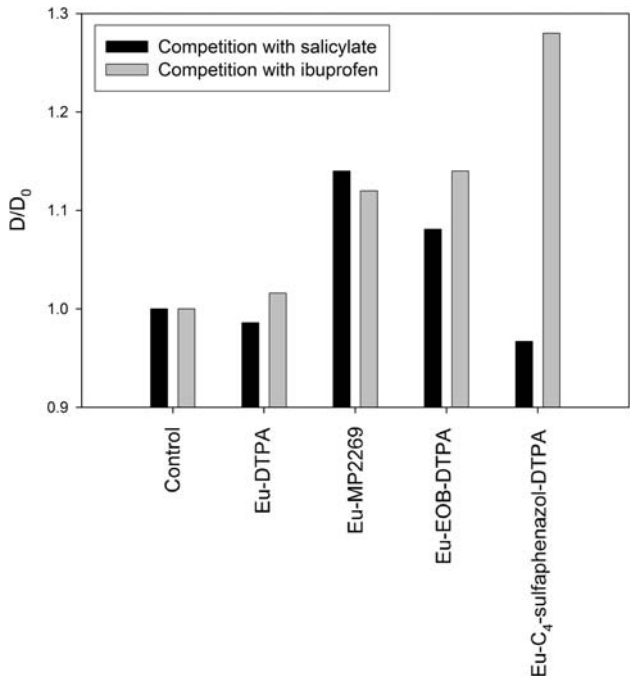

Fig. 6 Competition experiments with ibuprofen and salicylate performed in deuterated phosphate buffer at 200 MHz. The control corresponds to the diffusion coefficients of the competitors (D_0 for ibuprofen is 3.08×10^{-10} m^2 s^{-1} and D_0 for salicylate is 5.34×10^{-10} m^2 s^{-1}) in HSA solution, which were normalized to 1. The next *bars* correspond to the relative diffusion coefficients of the competitors in the presence of HSA and of 2 mM concentration of each europium chelate

for HSA. In the presence of Eu-MP2269 and Eu-EOB-DTPA, ibuprofen and salicylate are both displaced (D for ibuprofen increases from 3.08×10^{-10} to 3.45×10^{-10} m^2 s^{-1} with Eu-MP2269 and to 3.51×10^{-10} m^2 s^{-1} with Eu-EOB-DTPA; D for salicylate increases from 5.34×10^{-10} to 6.10×10^{-10} m^2 s^{-1} with Eu-MP2269 and to 5.80×10^{-10} m^2 s^{-1} with Eu-EOB-DTPA), showing that these two chelates probably interact on Suddlow sites I and II of HSA. In contrast, in the presence of Eu-C_4-sulfaphenazol-DTPA, only ibuprofen is displaced from its binding site(s) (D for ibuprofen increases from 3.08×10^{-10} to 3.94×10^{-10} m^2 s^{-1}), which means that this chelate seems to interact mainly on Suddlow site II of HSA. However, the presence of secondary binding sites on HSA complicates the analysis and it is, at this point, impossible to know if the europium chelates displace the competitors from their principal or their secondary binding sites. Therefore, our results would have to be confirmed by other techniques and/or by the use of other competitors.

These experiments are, however, attractive since only one measurement provides qualitative information about the affinity of a molecule and about its binding site.

Conclusions

These NMR diffusometry experiments confirmed the affinity of some chelates for HSA, as previously demonstrated by proton relaxometry. Both methods, however, suffer from some imprecisions on the quantitative data obtained. Indeed, in the diffusometry technique, the measured diffusion coefficients suffer from a relatively large error due to the presence of the HSA background in the NMR spectra. In contrast, in the proton relaxometry method, it is the impossibility of determining independently the relaxivity of the bound contrast agent which gives rise to some imprecisions.

In comparison with proton relaxometry, the main drawback of the NMR diffusometry technique is the duration of the measurements inherent to the low sensitivity of NMR, especially at a low concentration of the chelate. For instance, 1 week was necessary to perform the titration experiment for one europium or lanthanum

chelate. In addition, the quantities of substances used is slightly higher than those used in proton relaxometry.

In this study, the main advantage of NMR diffusometry is the additional information obtained about the exchange kinetics. In addition, the competition experiments gave straightforward information about the binding site of the ligand.

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