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Protein-Targeted Gadolinium-Based Magnetic Resonance Imaging (MRI) Contrast Agents: Design and Mechanism of Action

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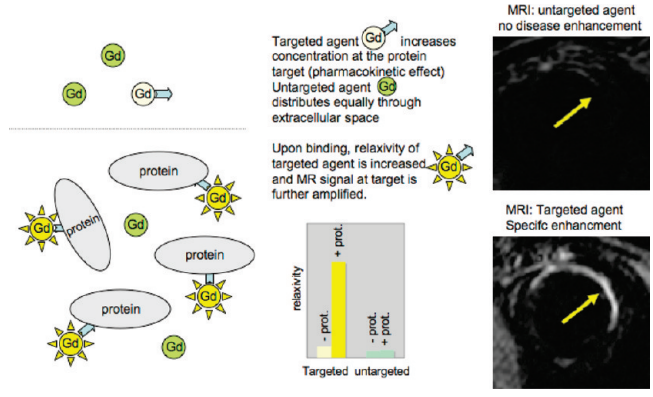

Magnetic resonance imaging (MRI) is a powerful medical diagnostic technique: it can penetrate deep into tissue, provide excellent soft tissue contrast with sub-millimeter resolution, and does not employ ionizing radiation. Targeted contrast agents provide an additional layer of molecular specificity to the wealth of anatomical and functional information already attainable by MRI. However, the major challenge for molecular MR imaging is sensitivity: micromolar concentrations of Gd^{III} are required to cause a detectable signal change, which makes detecting proteins by MRI a challenge.

Protein-targeted MRI contrast agents are bifunctional molecules comprising a protein-targeting moiety and typically one or more gadolinium chelates for detection by MRI. The ability of the contrast agent to enhance the MR image is termed relaxivity, and it depends upon many molecular factors, including protein binding itself. As in other imaging modalities, protein binding provides the pharmacokinetic effect of concentrating the agent at the region of interest. Unique to MRI, protein binding provides the pharmacodynamic effect of increasing the relaxivity of the contrast agent, thereby increasing the MR signal. In designing new agents, optimization of both the targeting function and the relaxivity is critical.

In this Account, we focus on optimization of the relaxivity of targeted agents. Relaxivity depends upon speciation, chemical structure, and dynamic processes, such as water exchange kinetics and rotational tumbling rates. We describe mechanistic studies that relate these factors to the observed relaxivities and use these findings as the basis of rational design of improved agents. In addition to traditional biochemical methods to characterize ligand−protein interactions, the presence of the metal ion enables more obscure biophysical techniques, such as relaxometry and electron nuclear double resonance, to be used to elucidate the mechanism of relaxivity differences.

As a case study, we explore the mechanism of action of the serum-albumin-targeted angiography agent MS-325 and closely related compounds and show how small changes in the metal chelate can impact relaxivity. We found that, while protein binding generally improves relaxivity by slowing the tumbling rate of the complex, in some cases, the protein itself can also negatively affect hydration of the metal complex and/or inner-sphere water exchange. Drawing on these findings, we designed next-generation agents targeting albumin, fibrin, or collagen and incorporating up to four gadolinium chelates. Through judicious molecular design, we show that high-relaxivity complexes with high target affinity can be realized.

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MRI Contrast Agents

Magnetic resonance imaging (MRI) is a powerful diagnostic technique: it can penetrate deep into tissue (unlike optical approaches), provide excellent soft tissue contrast with submillimeter resolution on clinical scanners (far better than nuclear imaging techniques), and does not employ ionizing radiation (like γ- and X-ray imaging). Targeted contrast agents add molecular specificity to the rich anatomical and functional information already attainable by MRI.

The major challenge for molecular MR imaging is sensitivity. In clinical MRI, water is imaged and contrast arises because of differences in water content among tissues or differences in the relaxation times T_1 and T_2 of water hydrogen nuclei. Contrast agents catalytically shorten T_1 and T_2 of water molecules encountering the agent. Despite high relaxation efficiencies, micromolar concentrations of Gd^{III} are required to cause detectable T_1 change in 55 M bulk water. This concentration requirement makes detecting proteins by MRI a challenge, but the attributes of MRI (resolution, no radiation, and shelf-stable contrast agents) make this challenge worth meeting.

Relaxivity (r_1 or r_2) is the change in the relaxation rate ($\Delta(1/T_{1\text{ or }2})$ normalized to the concentration of the agent in millimolar. The observed relaxation rate is given by eq 1

$$\frac{1}{T_i} = \frac{1}{T_i^0} + r_i[\mathrm{Gd}];\ i = 1, 2 \qquad (1)$$

where T_i^0 is the relaxation time in the absence of a contrast agent. The signal is proportional to $1/T_1$ and thus depends upon both the concentration of the agent *and* its relaxivity. This differs from nuclear and X-ray agents, which only depend upon the concentration.

Design Criteria

Contrast agents are administered intravenously and should have high aqueous solubility and be well-tolerated and inert with respect to metal loss, and the gadolinium must be completely eliminated from the body after the diagnostic test. Chart 1 shows various contrast agents discussed in this Account. These are all highly soluble, neutral, or anionic complexes that contain a multidentate ligand to sequester the potentially toxic gadolinium ion and which typically have open coordination sites for exchangeable water ligands. With notable exceptions,[1,2] the multidentate ligand is usually based on a cyclen or diethylenetriamine core with additional oxygen (e.g., acetate, amide, and phosphonate) or nitrogen (amine and pyridyl) donor atoms. Similar to other drugs, the development of targeted contrast agents involves optimizing the affinity and specificity of the agent for its protein target. Because the detection sensitivity is low, we must also optimize the relaxivity. The dual medicinal chemistry challenge of identifying compounds with both high target affinity and high relaxivity makes contrast agent development unique. This Account focuses on understanding and optimizing the relaxivity of targeted agents. Protein binding plays an enormous role, mostly positive but sometimes negative, in determining relaxivity. We describe tools to understand the mechanism of action and demonstrate strategies for high-affinity- *and* high-relaxivity-targeted contrast agents.

Serum Albumin Targeting with MS-325

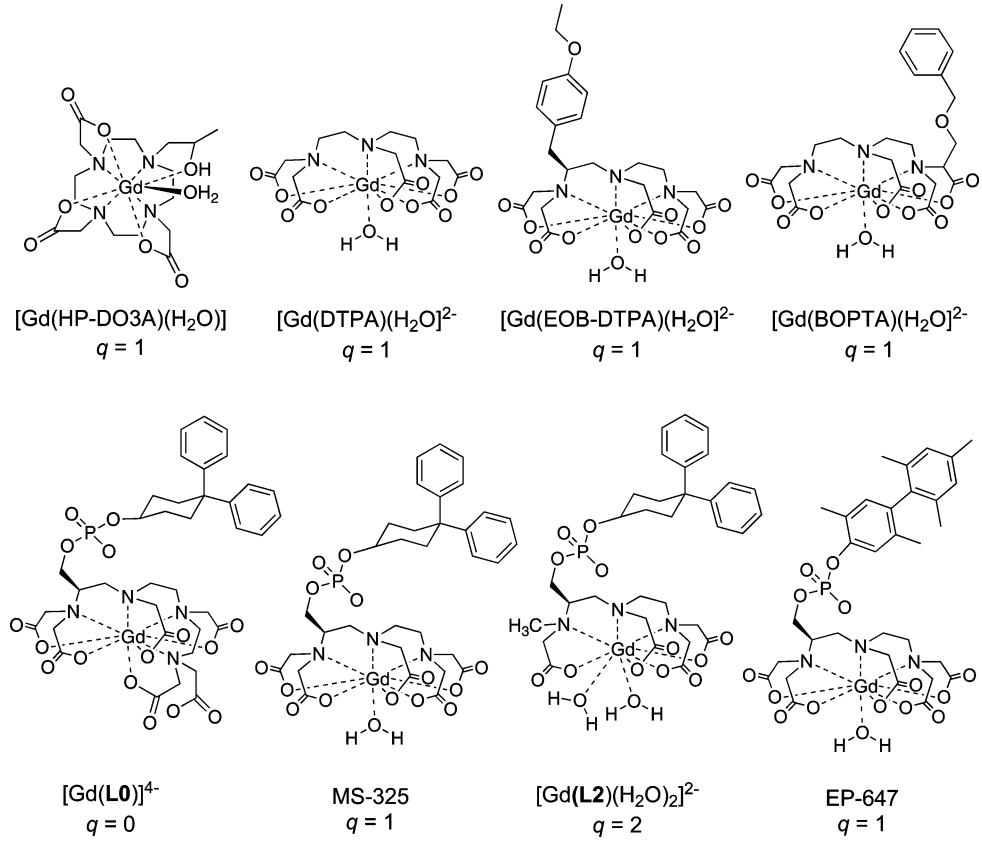
MS-325 (Chart 1) is an approved serum-albumin-targeted agent specifically designed for blood vessel imaging.[3] The Gd(DTPA)(H_2O) core (DTPA = diethylenetriaminepentaacetate) was modified to attach a biphenylcyclohexyl moiety via a phosphodiester linkage.[4] This amphiphilic complex targets serum albumin,[5] the most abundant blood plasma protein. Noncovalent binding restricts the distribution of the agent to the blood vessels, allowing for high-resolution images of arteries and veins to be acquired[6] (Figure 1). Albumin binding "hides" the agent from being filtered through the kidneys and extends plasma half-life, enabling imaging of one or more vascular regions with a single injection.[3] Reversible binding results in a small free fraction that is steadily being eliminated through the kidneys; important because the non-essential Gd^{3+} may prove toxic if it is not eliminated.[7] In addition to governing pharmacokinetics, protein binding has a pharmacodynamic effect in terms of increasing the MR signal by increasing the relaxivity of the agent by up to 10-fold.[5]

Protein Binding and Relaxivity

We sought to understand this increased protein-bound relaxivity for MS-325 and to use these mechanistic findings to guide the design of new targeted agents. Slowing the rotational tumbling time increases relaxivity, and this formed the basis for Lauffer's original design of targeted MR agents.[8] The seven unpaired electrons of Gd^{III} create a strong local magnetic field that fluctuates at the rate at which the complex tumbles in solution. The closer that this fluctuation frequency is to the ^1H Larmor frequency, the more efficient the relaxation of the bound water protons. Protein binding slows tumbling of the gadolinium complex from giga- to megahertz and increases relaxivity. This effect was termed receptor-induced magnetization enhancement (RIME) and underscored the rational design of MS-325 for blood vessel imaging.

Slowing tumbling increases relaxivity but unearths other limiting effects. Besides tumbling, relaxivity depends upon

CHART 1. Compounds, with the Number of Metal-Bound Water Molecules (*q*), Discussed in This Account



applied field, inner- and second-sphere hydration, water exchange rates, ion−proton distances, and the electronic properties of the ion. For noncovalent protein binding, speciation is important because the observed relaxation rate will depend upon what fraction is protein-bound. In addition, MS-325 exists in solution as interconverting diastereoisomers. We took a systematic approach using a range of physical and chemical methods to provide direct insight into each parameter influencing relaxivity.

Speciation of MS-325

The stability constant for MS-325 ($10^{22.1}$ M^{-1}) was measured and found to be 6 times more stable at pH 7.4 than GdDTPA (conditional stability constant at pH 7.4 = $10^{18.94}$ for MS-325 versus $10^{18.17}$ for GdDTPA).[9,10] This improvement in stability was attributed to the backbone substituent, which acts to preorganize the ligand toward binding. The presence of the backbone substituent also renders MS-325 10−100 times more inert to Gd substitution compared to other lanthanide−DTPA systems. Laurent et al. also found that MS-325 and other backbone-substituted GdDTPA derivatives were more inert with respect to transmetalation by Zn^{2+} than GdDTPA.[11]

LnDTPA complexes form Δ and Λ wrapping isomers that interconvert in solution. MS-325 is derived from L-serine, resulting in a *R* configuration at methine carbon 2 (Figure 2). Complexation makes the central N chiral, and therefore, four possible diastereomers are possible: Δ-*R*,*R*, Δ-*R*,*S*, Λ-*R*,*R*, and Λ-*R*,*S*. A 1.8:1 ratio of Λ-*R*,*R*/Λ-*R*,*S* is observed.[12] The disfavored Δ configuration results in the bulky albumin binding group in an axial position on the chelate ring. The interconversion is slower than in GdDTPA or in other DTPA derivatives, presumably because of this large substituent. Interconversion is catalyzed by acid, making it possible to isolate pure diastereomers by maintaining pH > 7.

Binding to Serum Albumins

Serum albumin is an abundant blood protein that binds a wide variety of ligands, typically nonsaturably.[13] We used equilibrium ultracentrifugation to directly determine albumin binding.[5] There was little difference in affinity for the two diasteromers,[14] hereafter collectively referred to as MS-325. MS-325 binds to fraction V human serum albumin (HSA) with an initial dissociation constant of 85 μM.[5,14] Binding is nonsaturable, and there are at least three additional binding sites of lower affinity ($1 < K_d < 4$ mM). Using site-specific fluorescent probe displacement, the high-affinity MS-325 site is so-called site 2 on subdomain IIIA, which is also the binding site for the drugs diazepam, ibuprofen, and naproxen.

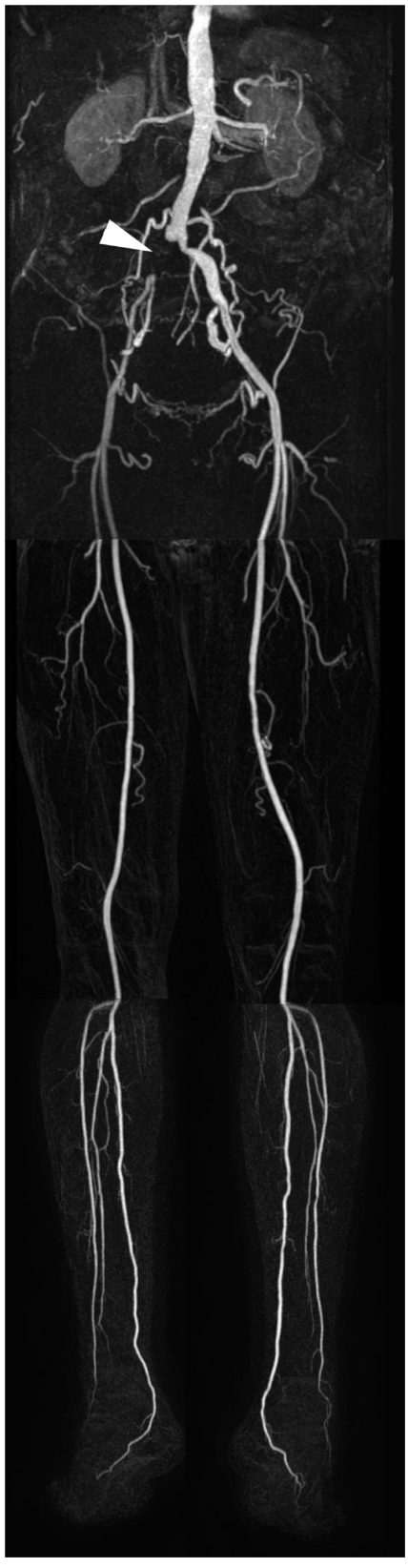


FIGURE 1. First pass image of the arterial tree immediately after injection of MS-325 demonstrates total occlusion of the right common iliac artery over its entire length (arrowhead). Image courtesy of Dr. Tim Leiner, University Hospital Maastricht.

In plasma, HSA has ≥2 long-chain fatty acids tightly bound, which affect the overall protein conformation. When fatty-acid-free HSA was used, the affinity for MS-325 was higher.[15] There was little difference in the MS-325 affinity for pig, dog, rabbit, rat, or mouse serum albumins. On the other hand, there were significant differences in plasma protein binding of MS-325 among these species.[15] Under standardized conditions of 0.1 mM MS-325 in plasma, the fraction bound ranged from 91.4% for human to 65.5% for rat. These differences could be understood when taking into account lower albumin concentrations in rats, mice, and dogs compared to those in humans or pigs. Such differences in albumin content result in MS-325 being less effective in rodent models than in humans. These studies also implied that albumin was the main target protein in plasma for MS-325.

Relaxivity

Figure 3 shows a nuclear magnetic relaxation dispersion (NMRD) profile, relaxivity as a function of field, for MS-325. Relaxivity is dramatically increased when the complex is bound to protein (part B versus A in Figure 3). In addition to the exchangeable water molecule in the inner-coordination sphere, other exchangeable water molecules contribute to relaxivity. The second-coordination sphere consists of counterions and water molecules that can also be relaxed and exchange with the bulk. Qualitatively, these curves are well-understood using the Solomon−Bloembergen−Morgan (SBM) theory.[16,17] Unfortunately, there are too many parameters to independently fit these curves. For q water molecules in the inner sphere, one requires the lifetime (τ_m) of these water molecules, a description of rotational motion (τ_R if isotropic, at least two more parameters if there is internal motion), and a description of electronic relaxation [Δ_t, a measure of transient zero-field splitting (ZFS) induced by solvent collisions, and τ_v, a correlation time for this modulation]. There is a sizable contribution to relaxation from water molecules outside the inner sphere that is dependent upon the number of water molecules, their lifetime, and the Gd−H distance. Finally, SBM is a high-field theory, in that it assumes a pure S state for the Gd^{3+} ion with no ZFS. However, all Gd complexes have measurable ZFS ($D = 0.02-0.06$ cm^{-1}).[18] Because NMRD probes very low Zeeman energies (at 10 kHz, the Zeeman energy is 100 times lower than the ZFS energy), at low fields, the SBM theory is not valid and more complex theories that take into account this static ZFS must be used. To use the NMRD data effectively, we sought to measure many of these parameters directly and unambiguously.

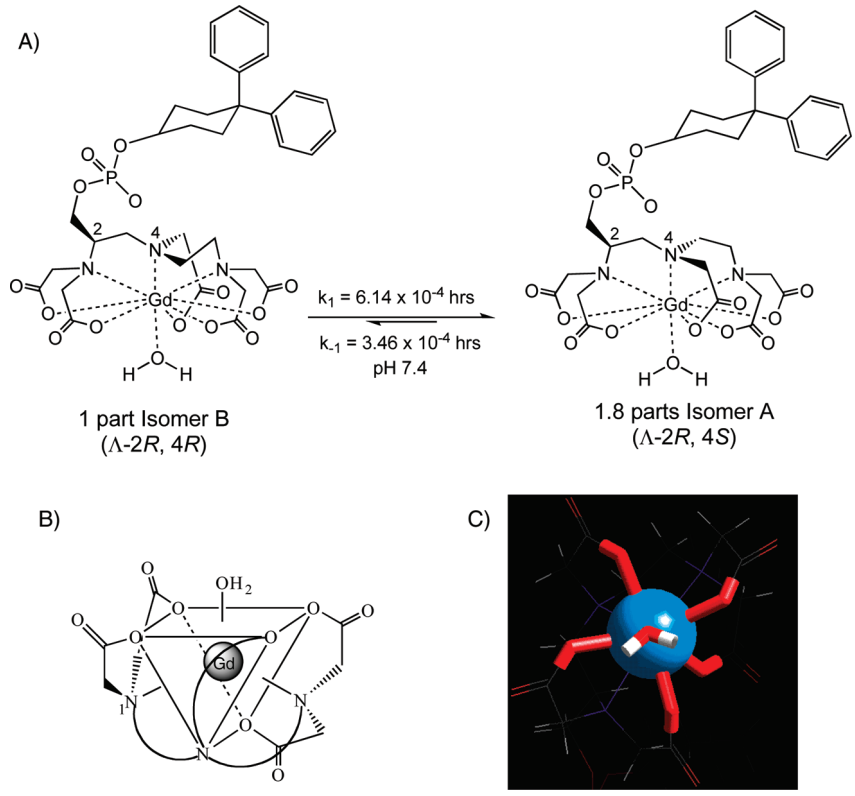


FIGURE 2. (A) Interconvertible diastereomers of MS-325. (B) Idealized tricapped trigonal prism (TTP) geometry of the complexes. (C) Looking down the water−Gd axis, highlighting the wrapping orientation.

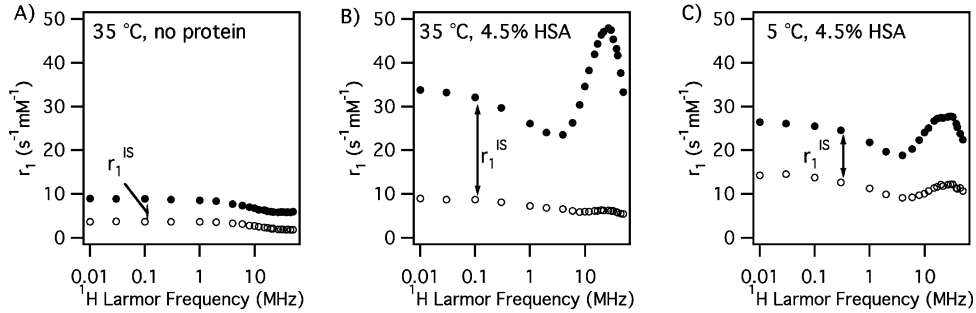


FIGURE 3. Relaxivity of MS-325 (●) and $q = 0$ Gd**L0** (○). The difference between the two is an estimate of the relaxivity due to the inner-sphere water molecule (r_1^{IS}) in MS-325: (A) 35 °C with PBS at pH 7.4, (B) 35 °C with 4.5% (w/v) HSA, and (C) 5 °C with 4.5% (w/v) HSA.

Second- and Outer-Sphere Hydration

Compounds with no inner-sphere water molecules can have significant relaxivity, and this increases upon protein binding.[19] The effect is due to exchangeable protons or water molecules present in the second-coordination sphere, with a lifetime longer than the diffusion time constant. This is difficult to measure directly; therefore, a triethylenetetraaminehexaacetic acid (TTHA) analogue of MS-325 was prepared (Gd**L0**, Chart 1) that has no inner-sphere water ligand. The relaxivity of this compound is assumed to be reflective of relaxivity contributions from non-inner-sphere waters to the MS-325 relaxivity.[5] Figure 3 shows that the relaxivity of Gd**L0** is more than doubled when bound to protein (part A versus B in Figure 3) and increases further with decreasing temperature (Figure 3C). Note how large the contribution to relaxivity can be from water molecules in the second and outer sphere.

Gadolinium−Hydrogen Internuclear Distance

The dominant relaxation mechanism for T_1 is the r^{-6}-dependent dipolar interaction between the electron spin of the Gd^{III} and the nuclear spin of the water proton. Small changes in this distance would have a big impact on relaxivity, but this fundamental measure of distance is difficult to directly obtain experimentally. A wide range of Gd−H distances for the inner-sphere water ligand were reported using indirect methods. For

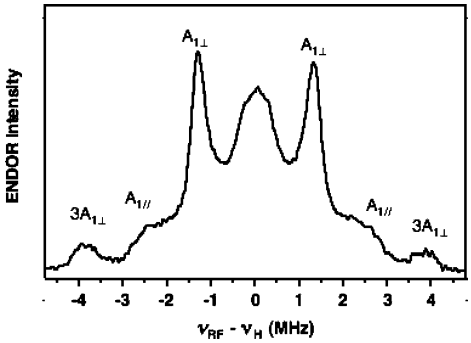


FIGURE 4. Mims ENDOR spectra of frozen solution (4.2 K) of $[Gd(H_2O)_8]^{3+}$ recorded at $B_0 = 5180$ G (maximum of $+^1/_2 \leftrightarrow -^1/_2$ transition in the EPR spectrum).

instance, after fitting NMRD curves, the substituted GdDTPA derivatives GdBOPTA, GdEOB-DTPA, and MS-325 (see Chart 1 for drawings of complexes) were reported to have Gd−H distances that were 0.2 Å shorter than the Gd−H distance in GdDTPA itself (2.9 versus 3.1 Å).[20,21] This amounts to a difference in relaxivity of 50% for this coordinated water molecule if all other parameters are equal. We were very intrigued by these reports of how modest changes to the ligand could profoundly reduce this distance. Even more surprising was the fact that the Gd−O(water) distance from X-ray crystallography was very similar for GdDTPA (2.44 and 2.49 Å), GdBOPTA (2.46 Å), MS-325 (2.48 Å), and other DTPA analogues.[12,22]

We collaborated with Arnold Raitsimring on a series of EPR studies to investigate this phenomenon. The electrostatic nature of bonding in lanthanide complexes results in very little electron spin delocalization onto the water hydrogen nucleus. The anisotropic hyperfine interaction, $T_\perp$, for this hydrogen atom is primarily determined by the dipole interaction with the electron spin on the Gd^{3+} ion. The Gd−H distance is simply related to $T_\perp$ by eq 2, where ρ is the spin density on the metal ion ($\rho \approx 1$) and other symbols are physical constants.

$$r_{GdH} \approx \sqrt[3]{\frac{g_e\beta_e g_n\beta_n\rho}{hT_\perp}} \qquad (2)$$

Pulsed Mims electron nuclear double resonance (ENDOR) for the Gd^{3+} aqua ion in frozen glassy solutions of gadolinium complexes is shown in Figure 4.[23] Positions marked $A_{1\perp}$ and $A_{1||}$ correspond to the perpendicular and parallel orientation, respectively, of the $\mathbf{r}_{Gd-H}$ vector with respect to the direction of magnetic field $\mathbf{B}_0$ for proton transitions within the $m_S = \pm^1/_2$ electron spin manifolds. From the position of these lines, we obtain $T_\perp$ and the isotropic hyperfine interaction, a_{iso}. This allows determination of the Gd−H distance with an accuracy

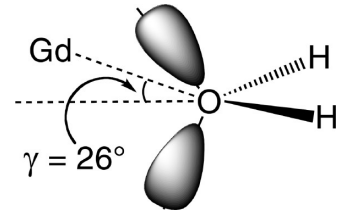


FIGURE 5. In contrast agents, the Gd−O bond does not lie along one of the lone pairs of a sp^3-hybridized oxygen. The Gd−O bond is oriented 26° with respect to the bisector of the two lone pairs on the oxygen.

of ±0.04 Å. The width of these lines is a measure of the distribution of distances.

For all of the Gd^{3+} complexes that we studied (11 published and additional unpublished data), there was no significant difference in the Gd−H distance.[18,23−25] We surveyed compounds of different charge, different donor set, eight- or nine-coordinate Gd^{III}, and macrocyclic versus linear coligands. In all cases, this distance was 3.1 Å and was distributed within the limits 3.0−3.2 Å; i.e., the bound water has a range of orientations. ENDOR of GdDTPA, MS-325, and GdBOPTA indicated no difference in the hyperfine interactions, demonstrating that there was no shortening of the Gd−H distance.[23] Our initial measurements[23] made at the K_u band (14.4 GHz) were confirmed by higher frequency 1D and 2D ENDOR measurements.[18,24]

Combining ^{1}H ENDOR with ^{17}O ENDOR to access the Gd−O distance[26] allowed us to develop a model of Gd−water coordination. Unlike what would be predicted from freshman chemistry, we found that the water is not oriented along one of the two lone pairs in a sp^3-hybridized oxygen. Such geometry would result in much shorter Gd−H distances than was observed. The water is oriented as in Figure 5 with the angle γ between the Gd−O and the bisector of the two lone pairs, equal to 26° and distributed within the range $0° < \gamma < 55°$, which is similar to what was observed in a solution neutron diffraction study.[27]

Inner-Sphere Hydration Number, *q*

Hydration numbers are typically estimated using a surrogate ion approach. Horrocks and Sudnick showed that the inner-sphere hydration was directly proportional to the difference of fluorescent decay rates measured in H_2O and D_2O because the O−H (but not O−D) oscillator from bound water is a good quencher of Eu^{III} fluorescence.[28] It is assumed that a Gd^{III} complex has the same hydration number as its Eu^{III} analogue.

The amplitude of the $A_\perp$ feature in ENDOR is proportional to the number of protons at that distance to the ion. We developed a technique to measure q by recording the ENDOR spec-

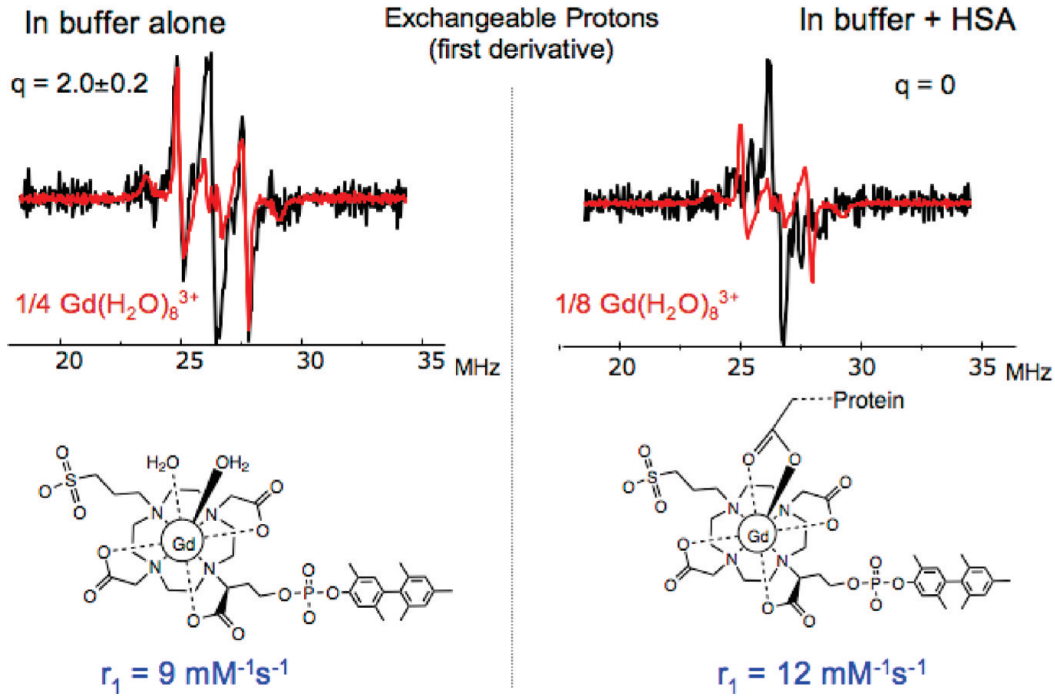


FIGURE 6. ENDOR method to estimate *q*. Take the difference spectrum of ENDOR in H_2O and D_2O to obtain the spectrum of exchangeable hydrogens (black trace) and compare the amplitude to the $Gd(H_2O)_8^{3+}$ spectrum (red trace). (Left) $q = 2$ complex with expected relaxivity. (Right) Relaxivity is much lower than expected when bound to albumin; ENDOR reveals no inner-sphere water.

trum in D_2O and H_2O.[25] The difference spectrum obtained gives the ENDOR of exchangeable protons. A consequence of the constant Gd−H distance is that the amplitude of this spectrum can be compared to that of the aqua ion ($q = 8$) to estimate the number of inner-sphere water ligands. ^{17}O ENDOR can be used similarly.[29] This provides direct access to the hydration state of the Gd^{III} ion and can be used for protein-bound complexes as well. MS-325 has a single coordinated water molecule in solution or when bound to serum albumin. For other complexes, protein binding can affect *q*. Figure 6 shows an ENDOR spectrum of an albumin-targeted complex that is $q = 2$ in solution but $q = 0$ when protein bound; presumably, water molecules are displaced by a carboxylate side chain on HSA.[25] Displacement of the inner-sphere water ligands mutes the relaxivity of the protein-bound complex.

Electronic Relaxation

The high-frequency EPR studies also provided insight into the crystal field interaction (cfi) parameters *D* and *E* for a range of Gd complexes. The cfi's are very important in determining relaxivity of Gd complexes, especially at low fields. At low fields, electronic relaxation is faster than the rate of molecular reorientation, resulting in proton relaxation being determined by *D* and the angle θ between the Gd−H vector and the *z* axis of the ZFS frame. At high fields, where the Zeeman energy is much higher than this ZFS energy, electronic relaxation is governed by transient distortions of the molecule to induce additional ZFS. The frozen solution high-frequency EPR studies provide insight into both of these effects in the presence or absence of the protein.[18] By simulating the EPR spectrum, one obtains estimates of the magnitude of *D* and the distribution of *D* values; i.e., there is not one value of *D* in frozen solution, rather *D* is statistically distributed between limits. This distribution is representative of the magnitude of the transient ZFS.

Inner-Sphere Water Exchange

Until the pioneering studies of the Merbach group,[30] it was widely assumed that water exchange at Gd^{III} complexes was extremely fast. We have since learned that this exchange rate can vary from 10^9 s^{-1} for the aqua ion[30] to $\sim 10^3$ s^{-1} for complexes, such as the DOTA−tetraamides.[31] Water exchange is typically measured from the temperature dependence of $H_2{}^{17}O$ relaxation in the presence and absence of the Gd^{III} complex. This technique requires millimolar [Gd] and lacks sensitivity for working with complexes bound to proteins, where protein solubility and stability limits the concentration and temperature range achievable. Usually, water exchange is measured in the absence of protein and assumed to not change when the complex is bound to protein.

The substitution of an acetate arm on MS-325 with a methyl group to give Gd**L2** (Chart 1) had a curious effect on

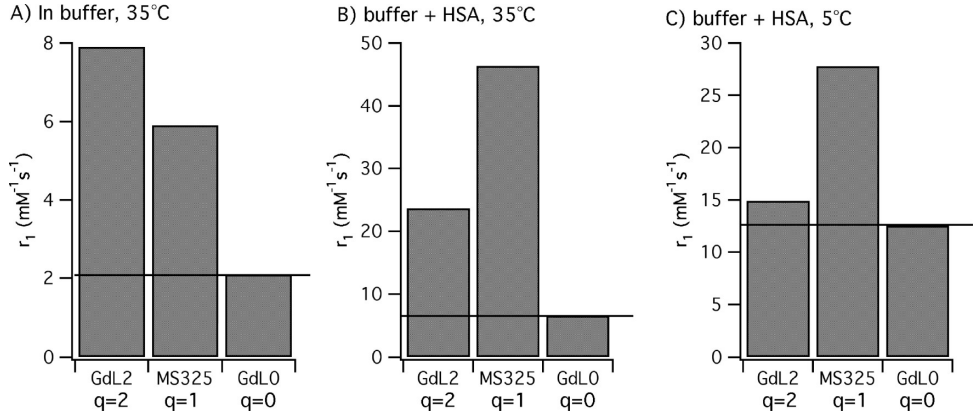


FIGURE 7. Two water molecules are not always better than one. (A) Relaxivity (20 MHz) of Gd**L2** is higher than MS-325 in buffer because of a larger q value. (B) In HSA solution, r_1 for $q = 2$ Gd**L2** is much lower than MS-325 because of slow water exchange. (C) Water exchange slows further at 5 °C; now, r_1 for Gd**L2** approaches that of Gd**L0**, a compound with no inner-sphere waters. Note the different scales.

relaxivity.[32] Removal of the acetate donor opened up an additional coordination site, and ^{1}H ENDOR measurements confirmed that Gd**L2** was $q = 2$ when bound to HSA. Figure 7 shows the relaxivities of Gd**L0** ($q = 0$), MS-325 (q=1), and Gd**L2** ($q = 2$) in PBS and HSA solution. In buffer alone, the relaxivities increase with increasing q as expected, and the inner-sphere contribution from Gd**L2** is about double that of MS-325. However, when HSA was added, Gd**L2** had significantly *lower* relaxivity when bound to protein than MS-325. Water exchange measurements showed that the water ligands at Gd**L2** underwent exchange 10-fold slower than water molecules bound to MS-325. Equation 3 points to the cause of this paradox in relaxivities. Observed relaxivity is factored into contributions from inner-sphere (IS), second-sphere (SS), and outer-sphere (OS) water molecules.

$$r_1^{obs} = r_1^{IS} + r_1^{SS} + r_1^{OS} = \frac{q/[H_2O]}{T_{1m} + \tau_m} + r_1^{SS} + r_1^{OS} \quad (3)$$

Assuming the latter two contributions are the same for these similar compounds, doubling q will double the inner-sphere contribution to relaxivity provided that T_{1m} and τ_m are the same. T_{1m} (the T_1 of the metal-bound water molecule) is similar for both compounds because the Gd−H distance is the same and both compounds bind similarly and to the same site on albumin, giving them similar rotational dynamics. In the absence of protein, T_{1m} is long (10 μs) relative to the lifetime of the water in the inner sphere, such that $T_{1m} > \tau_m$. Under these conditions, relaxivity is insensitive to water exchange and r_1^{IS} doubles with q. When bound to protein, T_{1m} becomes much shorter (<1 μs) and the denominator can be dominated by the water residency time τ_m. For HSA-bound MS-325, T_{1m} is still longer than τ_m and water exchange does not limit relaxivity at 37 °C, but for HSA-bound Gd**L2**, $\tau_m > T_{1m}$ and relaxivity is crippled by slow water exchange. It is unclear why water exchange at Gd**L2** should be slower than at MS-325. Interestingly, if the acetate arm on the central nitrogen of DTPA is substituted by a benzyl group to give a $q = 2$ complex,[33] then the water exchange rate is 20 times faster than Gd**L2**. These findings underscore how subtle changes to the ligand structure can significantly alter water exchange kinetics and relaxivity.

Nuclear Magnetic Relaxation Dispersion (NMRD)

The NMRD profile is very sensitive to rotation (tumbling) and whether there are internal motion contributions to relaxivity. Figure 8 shows NMRD of the two MS-325 diastereomers in HSA as a function of the temperature. The solid lines are fits to the data.[14] The model used the relaxivity of Gd**L0** to estimate $r_1^{SS} + r_1^{OS}$. The fitted parameters are in excellent agreement with estimates from other methods. Because r_1 decreases with temperature, relaxivity is very sensitive to τ_m and the water exchange rates estimated from NMRD are very similar to those obtained from ^{17}O NMR on the isomers in the absence of protein. We collaborated with Claudio Luchinat and Giacomo Parigi, who have developed a model that includes the effect of static ZFS on ^{1}H relaxation.[14,34] In concordance with our high-field EPR studies,[18] NMRD analysis showed similar ZFS parameters for both isomers and that the transient ZFS energy (distribution in D) was half as large as the static ZFS (D).[14] We found a rotational correlation time of 5 ns at 35 °C, which is long compared to the unbound MS-325 (0.13 ns) but still short compared to the correlation time of HSA. This discrepancy is likely due to two effects: motional flexibility of the molecule within the HSA binding pocket and also rapid rotation of the coordinated water ligand about the Gd−O axis.[35] This number is in agreement with other estimates of the cor-

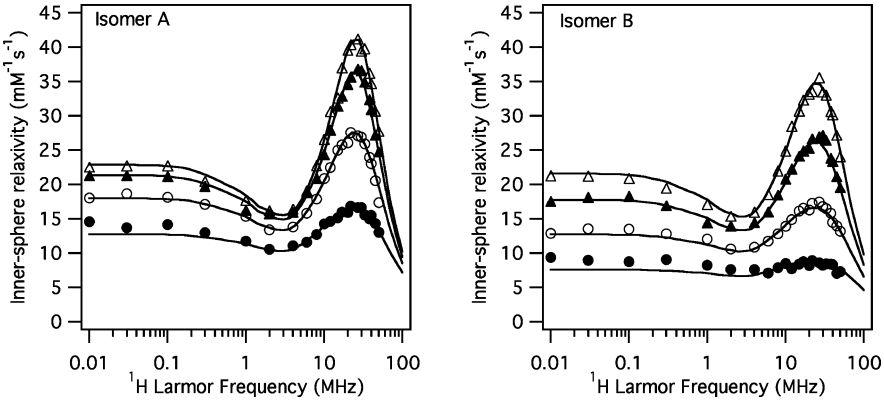


FIGURE 8. Temperature-dependent (△, 35 °C; ▲, 25 °C; ○, 15 °C; ●, 5 °C) NMRD of MS-325 isomers. Solid lines are fitted data using parameters described in the text.

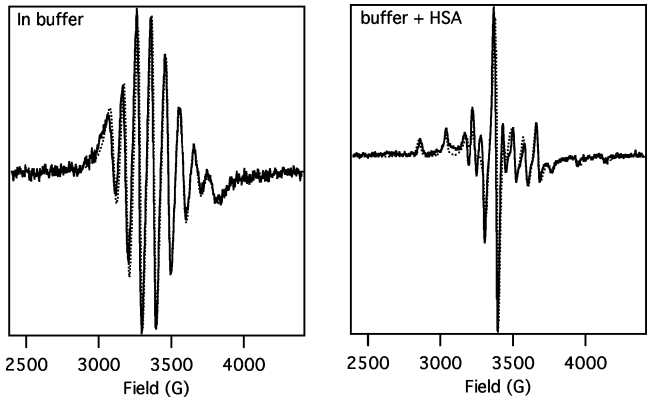


FIGURE 9. X-band EPR spectra of VO^{2+}-substituted MS-325 at 21 °C in buffer in the absence (left) or presence (right) of 4.5% HSA. Dashed lines are fits to the data.

relation time using surrogate methods, *vide infra*, and also from other NMRD estimates.[5,20]

Surrogate Methods To Probe Contrast Agent−Protein Interactions

Gd^{III} is easily replaced with another metal ion. As in inorganic biochemistry, surrogate ions with unique magnetic or optical properties can further probe the biophysics of relaxation, e.g., the Eu^{3+} for Gd^{3+} substitution to determine q as described above. Clarkson and colleagues substituted the vanadyl ion, VO^{2+}, for Gd^{3+} and estimated rotational correlation times from EPR.[36] Unlike Gd^{3+}, the EPR spectrum of VO^{2+} is strongly modulated by rotation. In collaboration with Prof. Clarkson, we made EPR measurements of the VO^{2+} version of MS-325 in the presence and absence of HSA (Figure 9). Simulating these spectra results in correlation times of 7.6 ns for the protein-bound complex and 0.188 ns for the unbound complex at 21 °C, in good agreement with 5.7 and 0.17 ns estimated from NMRD at this temperature. Because VO^{2+} only requires 5 of the 8 donor atoms on the DTPA chelator, the metal complex will certainly not be isomorphous with MS-325, but the good agreement in correlation times suggests that this is a useful

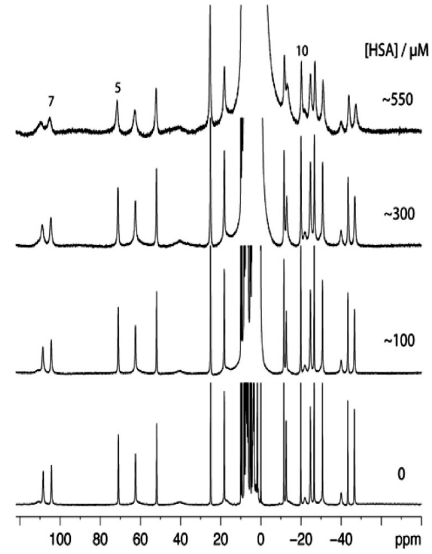


FIGURE 10. ^{1}H NMR spectrum of MS-325−Yb. Paramagnetic Yb^{3+} increases the chemical-shift dispersion but still results in an assignable spectrum. Measuring line broadening as a function of added protein allows for estimation of the rotational correlation time, τ_R, of the protein-bound complex.

methodology. The slightly higher correlation time for the vanadyl analogue could possibly be a result of an interaction between the uncoordinated arm of the ligand with the protein that further restricts motion.

Large but assignable pseudo-contact shifts describe the ^{1}H NMR spectrum of the Yb^{III} complex of MS-325. In albumin solution, where protein protons dominate the diamagnetic ^{1}H NMR region, the shifted resonances of the Yb−MS-325 surrogate are still observable (Figure 10).[37] With the aid of the MS-325 crystal structure,[12] we could estimate the Yb−H distances for the various protons. NMR spectra were recorded at increasing concentrations of albumin, resulting in increased line broadening. Because the metal complex−protein system was in fast exchange, the line widths were extrapolated to obtain line widths for the protein-bound species. These line

widths afford T_2, which is determined by the rotational correlation time and the Yb−H distance. Analyzing five different protons on the MS-325 ligand gave $\tau_R = 8 \pm 1$ ns for this complex bound to HSA at 37 °C, in good agreement with the numbers reported above.

Surprisingly, the relaxivity of MS-325 bound to rabbit, rat, or mouse albumin was markedly lower than for MS-325 bound to human, pig, or dog albumin.[15] We showed that q determined for the Eu^{3+} analogue ($q = 1$) did not change when bound to any of these proteins. NMRD of Gd**L0** indicated that there was no difference in second-sphere relaxivity in HSA or rabbit serum albumin. The Yb−MS-325 analogue indicated that τ_R was actually longer (13 ns) when bound to rabbit albumin, implying that relaxivity should be *higher* and not lower. This left an altered water exchange rate as a possible source of the relaxivity discrepancy. At high fields, Dy−MS-325 has a strong effect on T_2 relaxation of water protons because of Curie spin relaxation that is dependent upon τ_R and water exchange.[38] The higher sensitivity of ^{1}H relaxation allowed us to work under conditions where the protein was in excess and the metal complex was >90% protein-bound. Variable-temperature T_2 measurements unequivocally demonstrated that water exchange at the dysprosium ion was 9 times slower when the complex was bound to rabbit albumin than when it was bound to human albumin. Slower water exchange explained the lower relaxivity of MS-325 when bound to rabbit, rat, or mouse albumin. This water exchange effect is binding-site-dependent. EP-647, with a different biphenyl-albumin-targeting group (Chart 1), binds to a different site on albumin than MS-325. The relaxivity of EP-647 is the same when bound to rabbit or human serum albumin.[39]

The origin of the reduced water exchange rate for MS-325 binding to certain albumins is unknown. However, Baranyai et al.[40] nicely showed how alterations in the hydrogen-bonding network in the second-coordination sphere can alter water exchange rates in the inner sphere, and similar effects may be at play in our case.

Targeted Agents with Gadolinium Multimers

Image contrast is derived from T_1 change; this depends upon the product of relaxivity and the paramagnetic ion concentration, $\Delta(1/T_1) = r_1[\mathrm{Gd}]$. One can increase the Gd payload as well as its relaxivity. We take a small-molecule approach that has benefits of (1) a well-defined structure and reproducible synthesis, (2) elimination of the potentially toxic Gd via renal filtration of the chelate, and (3) rapid diffusion into interstitial space, useful for imaging nonvascular targets.

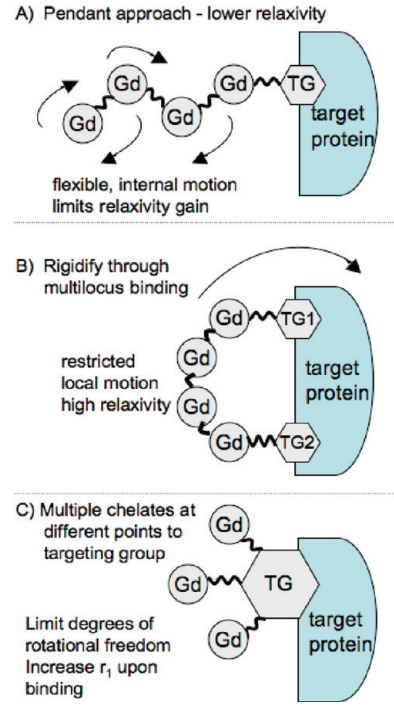


FIGURE 11. Three approaches to increase relaxivity. (A) Pendant approach increases Gd payload, but relaxivity gains are minimized because of internal motion. (B) Dual binder increases relaxivity by rigidifying the molecule upon binding. (C) Incorporate multiple chelates near the targeting group to minimize rotational degrees of freedom.

We have targeted the proteins fibrin (constituent of blood clots) and type I collagen (elevated in fibrosis).[41−44] Although these proteins are present at micromolar levels, multiple gadolinium complexes are needed to boost image contrast. To minimize the size and complexity of the molecule, high relaxivity remains a key goal. Rotational motion is one of the largest levers that we have to alter relaxivity, but as the molecule becomes larger, it is challenging to minimize internal motion. Figure 11 illustrates this problem and possible solutions. We reported two GdDTPA tetramers with either 1 or 2 HSA binding moieties.[45] In the absence of protein, both compounds show similar relaxivity. When the dual binder compound binds to HSA, the structure becomes rigidified and relaxivity increases, approaching 4 times that of MS-325. Because of flexible, internal motion that remains upon binding, the compound with one targeting group only achieves a bound relaxivity 2.5 times higher than MS-325, despite having 4 times more Gd. NMRD analysis indicated that the dual binding approach significantly decreased internal motion relative to the mono-binder. We have applied this dual targeting approach with a GdDTPA tetramer linked to fibrin-seeking peptides.[43] This approach has been used employing the same binding group that requires access to two similar binding sites. One can also envisage asymmetric compounds with one bind-

ing group to target a high-affinity site and a second group for a nonspecific hydrophobic interaction that serves to restrict rotation freedom.

An alternative approach with peptide targeting is to incorporate the Gd−chelates as close to the peptide as possible to minimize internal motion (Figure 11C). Rather than link a number of Gd−chelates to a targeting vector via a long flexible linker to minimize the impact of the chelates on protein binding, we advocate optimizing both relaxivity and affinity. This latter approach results in using fewer chelates per targeting group because the relaxivity is higher and may make the overall synthesis easier. The peptide-based fibrin-targeted agent EP-2104R incorporates two GdDOTA moieties at each of the C and N termini.[44] The peptide-based collagen-targeted probe EP-3533 has two GdDTPA moieties linked to the N terminus and an additional chelate in the middle of the peptide sequence.[41] In both instances, high relaxivities were observed.

Conclusions

Targeted MR contrast agents require optimization of both protein targeting and relaxivity. Relaxivity is a complex phenomenon that can be understood using a range of magnetic resonance and other physical methods. While protein binding often improves relaxivity through slowing the tumbling rate of the complex, the protein itself can also negatively affect hydration of the metal complex and inner-sphere water exchange. As a result, it is critical to synthesize and screen compounds with the protein of interest. One cannot assume that the properties of a given complex, e.g., hydration number or water exchange rate, will be maintained upon protein binding. Many papers on gadolinium complexes describe the potential for very high relaxivities ($r_1 > 100$ mM^{-1} s^{-1} per Gd) if rotational motion is completely restricted. Our experience and that of others is that such high relaxivities are not easily attained and that internal motion is a key limiting effect. However, there are effective strategies to reduce internal motion in targeted agents and to increase relaxivity through increased hydration number, fast water exchange, and second-sphere effects. Through judicious molecular design, high-relaxivity complexes with high target affinity can be realized.

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BIOGRAPHICAL INFORMATION

Peter Caravan received his B.Sc. (Honors) at Acadia University and his Ph.D. in chemistry from the University of British Columbia (under the guidance of Chris Orvig), followed by postdoctoral research at the Université de Lausanne (under the guidance of André Merbach). He spent 9 years at Epix Pharmaceuticals in Cambridge, MA, where he was ultimately responsible for all contrast agent research. He joined the Radiology faculty at Harvard Medical School and Massachusetts General Hospital in 2007. His research interests lie in development and application of molecular MR and PET imaging probes.

FOOTNOTES

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