Protein Relaxivity

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Multilocus Binding Increases the Relaxivity of Protein-Bound MRI Contrast Agents

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Dedicated to Professor Iwao Ojima on the occasion of his 60th birthday

The utility of clinical magnetic resonance imaging (MRI) has been greatly expanded by the use of contrast agents—gadolinium complexes that serve to increase the longitudinal nuclear relaxation rate ($1/T_1$) of water protons. Commercial contrast agents are only effective at high concentrations (> 0.1 mM) and, as a result, there has been considerable effort to increase their sensitivity. One approach is to increase the number of gadolinium ions in the molecule by making polymeric or dendrimeric contrast agents.[1] A second approach is to optimize the parameters that influence relaxation enhancement, such as rotational diffusion and water exchange.[2]

An effective mechanism for increasing relaxivity ($r_1 = (\Delta 1/T_1)/[\mathrm{Gd}]$) is the receptor-induced magnetization-enhancement (RIME) effect.[3] An example is the GdDTPA derivative MS-325, which targets serum albumin. When MS-325 binds to albumin, the rotational correlation time (τ_R) increases from that of a small molecule to that of the protein,

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Supporting information (synthesis of **1**, **2**, and **3**) for this article is available on the WWW under http://www.angewandte.org or from the author.

and the relaxivity increases ninefold to 51 $mM^{-1}s^{-1}$ (37 °C, 20 MHz).[4] This is shown schematically in Figure 1a and b. Despite this increased relaxivity, there is still a drive to increase relaxivity further to allow imaging of lower concentration targets. One approach for increased molecular relaxivity would be to combine the RIME effect with a multimeric gadolinium agent (Figure 1c). However, polymeric contrast agents such as GdDTPA-monoamide tethered to polylysine[5–8] or dextran[9,10] have much lower relaxivities ($r_1 < 13$ $mM^{-1}s^{-1}$ per ion at 37 °C) than one might expect based on their molecular weight because of internal motion along the polymer.[11] Furthermore, targeting multimeric contrast agents to a specific protein may also result in lower relaxivity than expected because of internal flexibility within the multimer.

It was hypothesized that a large RIME effect could be maintained if the multimer exhibited a certain rigidity when bound to the protein target. One approach would be to prepare a rigid multimer[12] by limiting the degrees of rotational freedom along the chain and then attaching a targeting vector to the multimer. A second approach in which a second targeting vector is used to anchor the terminus of the chain (multilocus binding) is outlined in Figure 1d. The second vector can be designed to bind nonspecifically to a hydrophobic patch on the target. In the absence of a target, there is rotational flexibility and low relaxivity; however, upon binding of the protein with both binding groups, the bound complex becomes more rigid and the relaxivity increases. In the absence of the second targeting group, the relaxivity of the bound species is lower because of internal flexibility. It is possible that the second targeting group could target a second protein which would also induce rigidity and enhance relaxivity.

To test this hypothesis, three tetrameric GdDTPA-based compounds were prepared (Figure 2) containing zero, one, or two protein-binding moieties. The DTPA moiety[13] shown in Figure 2 was used instead of the monoamide, because the GdDTPA complex has a more favorable water-exchange rate and the thermodynamically stable GdDTPA core is already present in several clinically approved contrast agents.[14] Human serum albumin (HSA) was chosen as the protein target for proof of principle and MS-325 (Figure 2) was used as a benchmark compound. Although MS-325 does not contain the same biphenyl albumin-binding group, it exhibits both high relaxivity and albumin binding and represents the state of the art.

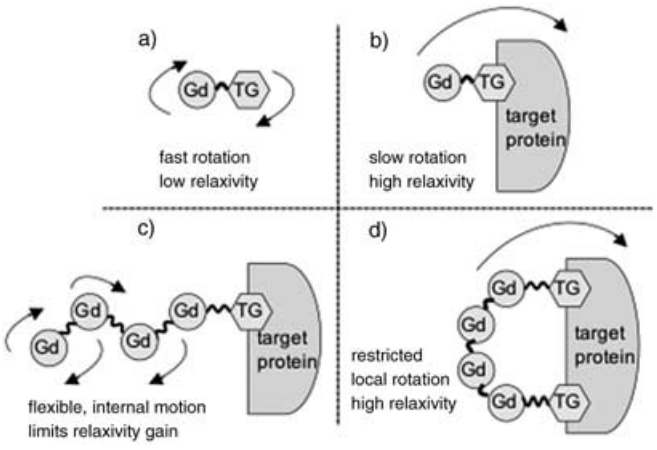

Figure 1. Strategies for increasing relaxivity: a) Discrete Gd chelate and targeting moiety, for example, the fast tumbling of MS-325 limits the relaxivity. b) Binding to the target slows tumbling, and relaxivity is increased. c) Gd chelate multimer with targeting; relaxivity may be limited by the fast internal motion. d) Gd chelate multimer with multilocus binding rigidifies the bound complex and increases the relaxivity.

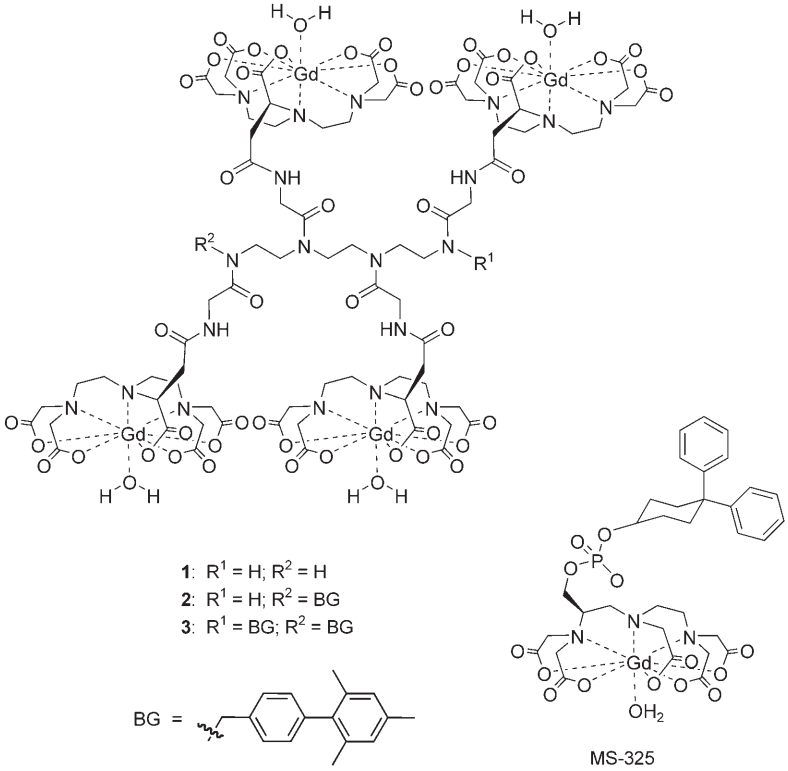

Figure 2. Compounds described in this study: GdDTPA tetramer containing zero (**1**), one (**2**), or two (**3**) HSA-binding groups (BG), and benchmark GdDTPA monomer MS-325.

The binding of each compound **1**–**3** (0.1 mM) to HSA (4.5% $w/v = 0.67$ mM) was assessed by ultrafiltration[4] through a 5-kDa-molecular-weight cut-off membrane at 37 °C and at pH 7.4; the gadolinium concentration in both the initial solution (total) and the filtrate was measured by inductively coupled plasma mass spectrometry (ICP-MS). Not surprisingly, compound **3** (with two binding groups) exhibits the highest affinity for HSA (Table 1). The relaxivities of **1**–**3** (0.1 mM) were measured at 20 MHz, pH 7.4, 37 °C in phosphate-buffered saline (PBS), in the presence or absence of HSA (0.67 mM). As a result of their increased molecular size, the relaxivities per Gd of the tetramers in buffer solution are higher than that of MS-325. When the measurement is made in an excess of HSA, the observed relaxivities follow the order **3** > **2** > **1**. To determine if this is simply a result of increasing albumin affinity, the relaxivity of the bound fraction can also be calculated ($r_1^{bd} = \{r_1^{obs} - f^{free} r_1^{free}\}/f^{bd}$). Table 1 shows that compound **3** has the highest albumin-

Table 1: Binding of MS-325, **1**, **2**, and **3**.[a]

Compound	Number of Gd	% bound	r_1^{obs} (PBS)	r_1^{obs} + HSA	r_1^{bd} (HSA)
MS-325	1	88	5.8 (5.8)	46 (46)	50.8 (50.8)
1	4	30	11.3 (45.2)	15.0 (60)	23.9 (95.6)
2	4	78	10.1 (40.4)	26.2 (104.8)	30.8 (123.2)
3	4	94	10.3 (41.2)	39.1 (156.4)	41.3 (165.2)

[a] Percentage of 0.1 mM compound bound to 0.67 mM HAS, observed per Gd relaxivities ($mM^{-1}s^{-1}$) at 20 MHz in the absence and presence of 0.67 mM HSA, and calculated albumin-bound relaxivities. Numbers in parentheses refer to relaxivity per molecule; all measurements were performed at pH 7.4, 37 °C, with 0.1 mM compound in PBS.

bound relaxivity of the three multimeric chelates. Although the GdDTPA moieties of **3** have a somewhat flexible linkage to the albumin-binding groups, the relaxivity per Gd of **3** in HSA approaches that of the more compact and rigid MS-325/HSA. On a per molecule basis, the relaxivity of **3** is clearly the highest.

To demonstrate that this result is indeed a rotational phenomenon, nuclear magnetic relaxation dispersion (NMRD) profiles were recorded for each of the three compounds, both with and without excess HSA at 35 °C. Figure 3 shows the relaxivity profiles for each compound

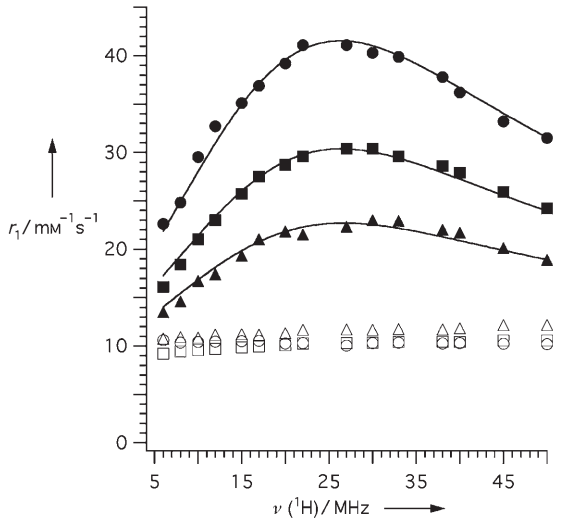

Figure 3. ^{1}H NMRD relaxivities expressed per Gd (35 °C, pH 7.4) of **1** (triangles), **2** (squares), and **3** (circles) (0.1 mM) in PBS only (open symbols) or bound to 4.5% HSA (filled symbols).

when bound to albumin (solid symbols, r_1^{bd}) or in buffer solution only (open symbols). The NMRD of the three compounds in buffer solution are similar and rather featureless, with an estimated rotational correlation time of about 0.3 ns. In the presence of excess albumin, however, the relaxivities of all three compounds are increased. Notably, the appearance of a high-field peak at about 25 MHz is typical of a slow-motion fraction.

The relaxivity in buffer solution and the HSA-binding data were used to calculate the bound relaxivity NMRD profiles. The high-field region is shown in Figure 3. Despite the complexity of the system (multiple chelates, complex rotational motion), it is informative to model these curves and this was performed in an approach previously described for the high-field part of the NMRD curve.[15] The NMRD of a $q=0$ triethylenetetraamine hexaacetic acid (TTHA) derivative reported earlier[4] was used to estimate the second- and outer-sphere contributions to relaxivity. The water residency time at 35 °C was fixed for all three compounds at 100 ns based on an analogy with other substituted GdDTPA compounds.[4,14] As the chelate part of the molecule is the same for all three compounds, it was assumed that the water-exchange rate would also be the same. The Gd—H(water) distance was fixed at 3.1 Å.[16] The three bound relaxivity NMRD data sets were fit simultaneously, the two parameters for electronic relaxation (Δ^2, τ_v) were treated as global parameters (owing to the identical chelating portions), and the rotational parameters were treated locally.

These data were fit in two ways: first, an isotropic model of rotation was employed to analyze these data. This model did not fit well—the peak shape was not well reproduced—and the fitted curve gave a much flatter peak that underestimated the relaxivity maximum and overestimated the relaxivity at higher fields. The correlation times from the fitting are in the 1-ns range and do not reflect the physical reality of an albumin tethered complex. The second method was a Lipari–Szabo model-free approach that has been described previously.[15] Equation (1) describes the dipolar

$$\frac{1}{T_{1M}} = \frac{C}{r_{GdH}^6}\left(\frac{3F\tau_{cg}}{1+\omega_H^2\tau_{cg}^2} + \frac{3(1-F)\tau_{cl}}{1+\omega_H^2\tau_{cl}^2}\right);\quad \frac{1}{\tau_{cg}} = \frac{1}{\tau_g} + \frac{1}{T_{1e}} + \frac{1}{\tau_m};\ \frac{1}{\tau_{cl}} = \frac{1}{\tau_{cg}} + \frac{1}{\tau_l} \tag{1}$$

relaxation of the coordinated water. In this case, the spectral density function has two terms that are related by a factor F (sometimes called an order parameter and denoted S^2), which describes the degree of spatial restriction. If the motion of the Gd chelate is independent of the protein, then $F=0$; if the chelate is completely immobilized on the protein, then $F=1$. There are two correlation times, τ_{cg} and τ_{cl}, which contain contributions from global (τ_g) and local (τ_l) rotational correlation times, as well as from the electronic relaxation time T_{1e}.

A global correlation time based on fluorescence anisotropy decay was fixed at 41 ns,[17] whereas the local correlation time for each compound was allowed to vary along with its order parameter. In this case, the peak shape is much better reproduced, and the resultant fitted parameters are given in Table 2. As may be expected, the order parameter F increases from compound **1** to **2** to **3** as the number of binding groups

Table 2: Fitted NMRD parameters for compounds **1**, **2**, and **3**.[a]

τ_v	Δ^2	**1**		**2**		**3**	
[ps]	[10^{18} s^{-2}]	F	τ_l [ns]	F	τ_l [ns]	F	τ_l [ns]
13	7.1	0.05	0.68	0.09	0.92	0.16	1.40
(1)	(0.2)	(0.01)	(0.03)	(0.01)	(0.03)	(0.02)	(0.04)

[a] Global (HSA) rotational correlation time fixed at 41 ns. Numbers in parentheses represent one standard deviation.

increase. The local correlation time also increases in the order **1** < **2** < **3**, which suggests that compound **3** is the most rigid when bound to albumin. Although the order parameters are relatively low, it was noted that they correlated with the global correlation time—arbitrarily chosen shorter global correlation times also gave good fits, but the order parameter was increased. Although the Lipari–Szabo approach models the data well, motion within this system is complex and care should be taken in comparing these rotational parameters with other unrelated systems.

There have been gains reported in increasing the relaxivity of single gadolinium complexes through the optimization of water exchange, hydration number, etc. Multilocus binding may prove to be a useful technique for maintaining this high relaxivity when these complexes are incorporated into targeted multimeric MRI contrast agents.

Experimental Section

The compounds **1**, **2**, and **3** were synthesized in an analogous fashion (see Supporting Information for full details). 4-Mesitylbenzoic acid was coupled to zero, one, or two of the primary amine groups of triethylenetetraamine. After reduction of the amide, the resultant tetraamine was coupled to four protected DTPA moieties. Deprotection, chelation, and purification by HPLC yielded the complexes shown in Figure 2. All Gd concentrations were determined by ICP-MS (Agilent 7500). NMRD profiles were recorded on a Koenig-Brown field-cycling relaxometer.

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