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Gd(DOTAla) – A single amino acid Gd-complex as a modular tool for high relaxivity MR contrast agent development

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Abstract

MR imaging at high magnetic fields benefits from an increased signal to noise ratio, however T_1 based MR contrast agents show decreasing relaxivity (r_1) at higher fields. High field, high relaxivity contrast agents can be designed by carefully controlling the rotational dynamics of the molecule. To this end, we investigated applications of the alanine analogue of Gd(DOTA), Gd(DOTAla). Fmoc protected DOTAla suitable for solid phase peptide synthesis was synthesized and integrated into polypeptide structures. Gd(III) coordination results in very rigid attachment of the metal chelate to the peptide backbone through both the amino acid sidechain and coordination of the amide carbonyl. Linear and cyclic monomers (GdL1, GdC1), dimers (Gd2L2, Gd2C2) and trimers (Gd3L3, Gd3C3) were prepared and relaxivities were determined at different field strengths ranging from 0.47T to 11.7T. Amide carbonyl coordination was indirectly confirmed by determination of the hydration number q for the EuL1 integrated into a peptide backbone, $q = 0.96 \pm 0.09$. The water residency time of GdL1 at 37 °C was optimal for relaxivity, $\tau_M = 17 \pm 2$ ns. Increased molecular size leads to increased per Gd relaxivity (from $r_1 = 7.5$ for GdL1 to $12.9 \text{ mM}^{-1}\text{s}^{-1}$ for Gd3L3 at 1.4T, 37 °C). The cyclic, multimeric derivatives exhibited slightly higher relaxivities than the corresponding linearized multimers (Gd2C2: $r_1 = 10.5 \text{ mM}^{-1}\text{s}^{-1}$ versus Gd2C2-red $r_1 = 9 \text{ mM}^{-1}\text{s}^{-1}$ at 1.4T, 37 °C). Overall, all six synthesized Gd complexes had higher relaxivities at low, intermediate and high fields than the clinically used small molecule contrast agent [Gd(HP-DO3A)(H₂O)].

Keywords

Gadolinium; Chelates; Molecular Imaging; Peptide; Magnetic Resonance Imaging

INTRODUCTION

Magnetic resonance imaging (MRI) is one of the most important modalities used for non-invasive investigation of disease in the clinic. MRI is the imaging technology of choice whenever high-resolution tissue contrast is required. Another advantage is the use of harmless magnetic fields for MRI as opposed to ionizing radiation in the case of CT.¹²

A large fraction of scans performed in the clinical setting are further enhanced by the use of contrast agents.³ Contrast agents shorten the relaxation times of water molecules in their proximity and increase tissue contrast on relaxation weighted imaging sequences. Currently, most clinically employed contrast agents are non-target specific, small molecule gadolinium complexes which are able to increase the longitudinal relaxation rate $1/T_1$ of water protons

in the extracellular space.⁴ The extent to which a contrast agent can enhance relaxation depends on its concentration and its relaxivity (r_1), an inherent property of the molecule. Most approved contrast agents have low relaxivities (r_1) which makes them effective only at relatively high concentrations (> 0.1 mM).⁴ There has been a considerable research effort to increase the relaxivity of contrast agents.^{5,6-9} Compounds with high relaxivity can be detected at lower doses,¹⁰ or provide greater contrast at equivalent dose to compounds with lower relaxivity. Additionally, an attachment of a targeting moiety allows for target specific delivery of the contrast.¹⁰⁻¹² The clinically approved blood pool agent MS-325 (gadofosveset, Ablavar) is an example of a contrast agent with a high relaxivity;^{13, 14} this small molecular compound carries an albumin-targeting moiety and will display an over 8 fold increase in relaxivity at low fields once it is associated with human serum albumin (HSA).¹⁵

While 1.5T remains the dominant field strength for clinical MRI, there is now a large installed base of 3T scanners and the major equipment vendors also offer 7T whole body human scanners. Small animal scanners operate almost exclusively at field strengths of 4.7T and higher. The primary benefit of high field is the increased signal to noise ratio, which enables greater spatial resolution and reduced acquisition time. In addition, the inherent T_1 of tissue increases with increasing magnetic field.¹⁶

Thus a contrast agent with equivalent relaxivity at a high and low field would provide much greater contrast at the high field. However the relaxivity of many T_1 -contrast agents decreases more rapidly with applied field than the inherent tissue T_1 increases.

Relaxivity above 0.1 T depends on a variety of parameters, some of which are depicted in Figure 1.¹⁹ As the magnetic field increases, the optimal correlation time, τ_c , for maximum possible relaxivity decreases, as it is inversely dependent on the proton Larmor frequency ω_H . While the contribution from the electronic relaxation time (T_{1e}) is negligible at fields above 1.5 T, contributions from the mean water residency time (τ_M) and the rotational correlation time (τ_R) become the levers for generating high relaxivity Gd-based agents.²⁰

For the design of high field, high relaxivity contrast agents it is instructive to consider the equation for two site exchange written in terms of inner-sphere water relaxivity, eq 1, and the Solomon equation, eq 2, which describes the field dependence of T_1 relaxation of the coordinated inner-sphere water hydrogen atoms.³ Equation 1 teaches that the inner-sphere water relaxation time T_{1M} and the water residency time, τ_M , should be as short as possible. With regards to T_{1M} , equation 2 indicates that the correlation time should be as large as possible, but while still meeting the requirement of $\omega_H \tau_c < 1$, where ω_H is the proton Larmor frequency and C is a constant. For a given Larmor frequency, there is an optimal correlation time. Unless water exchange is exceedingly fast ($> 10^9$ s⁻¹), the correlation time at 1.5T and higher will essentially be the rotational correlation time, τ_R . If τ_R is very long (nanoseconds and longer), then relaxivity will be very high at low fields, but the condition $\omega_H \tau_c > 1$ will also occur at lower fields and relaxivity will be low at high fields.

$$r_1^{Is} = \frac{q/[H_2O]}{T_{1m} + \tau_M} \quad (1)$$

$$1/T_{1m} = C \left[\frac{3\tau_c}{1 + \omega_H^2 \tau_c^2} \right] \quad (2)$$

For instance, MS-325 was designed for high relaxivity at low fields (1.5T). Serum albumin binding of MS-325 results in a very long τ_R resulting in high relaxivity at 1.5 T, but a precipitous decline in relaxivity with increasing field strength.^{21, 22} Small molecule agents with very short correlation times such as $[\text{Gd}(\text{DTPA})(\text{H}_2\text{O})]^{2-}$ (Magnevist) display a modest relaxivity decrease with increasing field strength but exhibit relatively low relaxivity due to their rapid tumbling.²³ We have previously investigated interplay of water exchange and rotational correlation time for Gd-based T_1 agents at fields ranging from 0.47 to 9.4 T,²¹ and showed that the optimal ranges are $5 < \tau_M < 25$ ns and $0.5 < \tau_R < 2$ ns to yield high relaxivity over a range of fields. A number of compounds with a corresponding, intermediate τ_R value between 0.35 – 1 ns has been reported,^{18, 24, 25} however none of these structures allow for the simple adjustment of τ_R without sacrificing rigidity of the Gd-complex or complete redesign of the entire scaffold.

This paper describes synthesis and investigation of a unique, modular system, capable of the construction of a new generation of high relaxivity T_1 contrast agents for high magnetic fields. Peptide structure and Gd complex incorporation can be easily modified using solid phase peptide synthesis, without change of the local complex environment.

STRUCTURAL DESIGN

Control and optimization of τ_R requires rigid attachment of the corresponding Gd complex to a molecular construct of appropriate size. Conjugating the Gd complex to a targeting vector or molecular scaffold is typically done through a single linkage, and this results in fast internal motion about that linkage and concomitant lower relaxivity. Tweedle and coworkers pioneered the dual anchor strategy,²⁶ which was also employed by Desreux and colleagues to rigidify attachment of the metal complex for the construction of fatty acid derivatized Gd(DOTA)²⁷. A similar, multi-site attachment strategy was employed for the design of metallostars, where a metallic barycenter is used as a point of attachment for multiple Gd(DTTA) type complexes (Figure 2, approach A).²⁴ As attachment of multiple copies of the Gd complex increases size, the enhancement of τ_R combined with increase of the Gd-complex payload will further expedite molecular relaxivity.^{25, 28, 29} Meade and colleagues employed click chemistry to attach multiple Gd complexes to rigid, all-organic barycenters (Figure 2, approach B).^{5, 25} Alternatively, Parker and coworkers showed that the rigid Gd complex can itself be placed at the barycenter of a molecule of variable size (Figure 2, approach C).¹⁸ Most of these constructs display enhanced relaxivity at high fields compared to systems with either very short or very long τ_R . We concluded that a combination of 1) rigid attachment of the metal complex using the dual anchor strategy, 2) multimerization and 3) easy adjustment of molecular size would provide a construct highly suitable for high field applications.

The immediate coordination environment around the Gd complex influences important parameters such as kinetic inertness and water exchange kinetics. While $q = 2$ complexes can provide great relaxivity enhancement due to two or more possible sites of interaction for water molecules with the paramagnetic metal,^{30–34} only few have the kinetic inertness with respect to Gd dissociation/transchelation required for *in vivo* applications. For $q = 1$, a myriad of kinetically inert Gd(DOTA) type complexes have been well characterized and provided us with the information necessary for choosing a suitable system.^{35, 36}

DOTA mono-protonamide derivatives, where the amide forms a 6-membered chelate ring upon coordination of Gd(III), were found to have a mean water residency time of 10–20 ns (at 37 °C), which is within the ideal range required for our purposes (Figure 3, compound E).³⁷ Geraldès and coworkers have reported the synthesis and investigation of Gd(DO3A-N- α -aminopropionate) (Figure 3, Compound F).³⁸ This system provided the basis for our

investigations. We reasoned that derivatization and multimerization of DO3A-N- α -aminopropionate could be achieved by using an Fmoc-analogue of this system and standard peptide synthesis, similar to an approach previously explored by Sherry and coworkers (Figure 3, compound G),³⁹ as well as by Stephenson et al.⁴⁰ In our system, the complex is linked to the peptide backbone via the short methylene linkage of alanine sidechain. Gadolinium coordination of the amide carbonyl, which is also used for coupling to the polypeptide, provides the second point of attachment and results in a rigid incorporation of the complex into the polypeptide that should restrict internal motion and enable control over τ_R . This design would be capable of satisfying all our criteria: fast water exchange, tunable rotational dynamics; limited internal motion, and ease of derivatization using solid phase synthesis. Moreover, by using Gd(DOTAla) moiety itself to increase molecular size, the overall molecular relaxivity is increased via multimerization (Figure 3, compound H).

RESULTS AND DISCUSSION

SYNTHESIS OF FMOC-DOTALA

For the use of DO3A-N- α -aminopropionate in solid phase peptide synthesis, construction of the corresponding Fmoc-derivative ("Fmoc-DOTAla-^tBu₃", compound **6**) was required. Fmoc is easily deprotected under mildly basic conditions while the ligandcarboxylates remain protected⁴¹ hence it is more suitable for our purposes than a potential Boc-derivative.⁵ As cyclen has high inherent basicity, the Fmoc protective group can only be introduced after alkylation of all secondary amines on cyclen.

We employed a synthetic strategy similar to the one used by Sherry and coworkers³⁹ in order to afford **6** in 15 % over all yield after 5 synthetic steps (Scheme 1). Commercially available serine derivative **1** was converted into the mesylate **2**. Our initial attempted introduction of this sterically crowded synthon onto commercially available *t*-butyl protected DO3A failed. Instead, **2** was used for the mono-N-alkylation of cyclen. Compound **3** was further alkylated after removal of excess cyclen mesylate salt using *tert*-butyl bromoacetate in order to afford compound **4**, which was isolated using preparative HPLC. Simultaneous removal of the benzyl and carboxybenzyl protective groups using H₂ and Pd/C yielded compound **5**. Using Fmoc-Cl in a mixture of H₂O and dioxane under basic conditions for **3** results in the introduction of the desired Fmoc protective group. Compound **6** is purified using preparative HPLC. It was found that using extended reaction times over 12h leads to a considerable amount of side products of which some are more difficult to separate from the final product.

Synthesis and evaluation of monomeric Metallopeptide—As a next step, we aimed to incorporate **6** into the linear model sequence H-Cys(Acm)-Gly-DOTAla-Gly-Phe-Cys(Acm)-CONH₂ (H₃L**1**, Scheme 2). The corresponding Gd(III) complex would allow us to study water exchange kinetics, while the analogous Eu(III) complex provides information on the hydration number (*q*) at the metal center. Rather forcing conditions encompassing HATU in the presence of NMM in DMF were required for significant product formation. We synthesized H₃L**1** using PEGA-Rink resin and standard manual solid phase synthesis (Scheme 2). Incorporation of a Phe residue was employed to provide a UV handle for detection and purification. Two Cys residues were used as the terminal amino acids, serving as potential sites of secondary structure modification through intramolecular disulfide bond formation. The *tert*-butyl esters were removed simultaneously with cleavage from the resin using a typical acidic cleavage cocktail (TFA: DDT: TIPS: H₂O (9.5:0.25:0.25:0.25)). The crude peptide H₃L**1** was isolated using ether precipitation. Complexation with either GdCl₃·6H₂O or EuCl₃·6H₂O by mixing of aqueous solutions of the metal salt and the crude peptide at pH 3, followed by slow adjustment of the pH to 6.5 using an aqueous 0.1 M

NaOH solution yielded the corresponding crude metal complex. The metallo-peptide was purified using preparative HPLC.

This class of Ln-DOTA derivatives is typically 9-coordinate. If the amide carbonyl from the peptide backbone was coordinated to the lanthanide, then we would expect a single aqua co-ligand, i.e. $q = 1$. The luminescence lifetime of the Eu(III) complex was measured in H₂O and D₂O. A custom-designed multimodal confocal imaging system built by Yaseen et al.⁴² was used to measure the luminescence lifetime of the Eu(III) excited state ⁵D₁ as previously reported.⁴³ Luminescence lifetimes were measured and averaged and used for the modified Horrocks equation⁴⁴ (equation 3), which accounts for the amide donor as one of the ligands.

$$q_{\text{H}_2\text{O}} = 1.2 \left[\left(\frac{1}{\tau_{\text{H}_2\text{O}}} - \frac{1}{\tau_{\text{D}_2\text{O}}} \right) - 0.325 \right] \quad (3)$$

The value obtained for hydration number q was 0.96 ± 0.09 , which suggests that the carbonyl of the peptide backbone is indeed coordinated to the metal ion.

Water exchange kinetics for the inner-sphere water ligand were determined by measurement of the temperature dependence of the transverse relaxation time T_2 of H₂ ¹⁷O in the presence and absence of GdL1. The data in Figure 4 were fit to a 4 parameter model as described previously.²² The water exchange rate at 310K, ³¹⁰ k_{ex} , its activation enthalpy, $\Delta H^\ddagger$, the electronic relaxation time at 310K, ³¹⁰ T_{1e} , and its activation energy, $\Delta E^\ddagger$ were iteratively varied to fit the observed reduced relaxation rate data R_{2r} . The hyperfine coupling constant was fixed at 3.8×10^6 rad/s.⁴⁵ At the high field used, τ_M dominates the scalar correlation time and results in an accurate estimate of water exchange, while the relative contribution of T_{1e} to ¹⁷O nuclear relaxation is much lower and this parameter is less well defined, Table 1. The water residency time ($\tau_M = 1/k_{\text{ex}}$) was determined to be 17 ± 2 ns at 310 K, which is similar to the Gd-DOTA-monopropionamide derivative reported by Geraldes and coworkers (Table 1, Figure 4).³⁸ This similar water exchange rate is also consistent with the amide carbonyl as a donor. We further note that this water residency time is in the optimal range for high relaxivity at all field strengths.

MULTIMERIC METALLOPEPTIDES

GdL1 demonstrated that the GdDOTAla moiety could be incorporated into a peptide, and the resultant complex had the expected single inner-sphere water co-ligand with an optimal water exchange rate. However GdL1 is still a rather small molecule with a relatively short τ_R . In order to increase τ_R and enhance the molecular relaxivity, we also synthesized dimeric and trimeric structures. The cysteines were either left protected ('linear' structures) or were deprotected and used to induce intramolecular cyclization ('cyclic' structures) in order to highlight the possibility of secondary structure modification with our approach (Scheme 2). Multimers H₆L2 and H₉L3 were furnished using the same synthesis methodology as for the linear, model monomer peptide H₃L1. On-bead deprotection of the Ac_m protective group on the Cys amino acids using I₂ was done in order to afford the cyclized analogues H₆C1, H₆C2 and H₉C3.⁴⁶ As cyclization was only 60 % complete for compounds H₆C2 and H₉C3, cyclization was driven to completion using 2% DMSO in H₂O at pH 8 (Scheme 2).⁴⁷ The Gd complexes GdL1, Gd₂L2 Gd₃L3, GdC1, Gd₂C2 and Gd₃C3 were formed and purified using the same methodology as described for the monomer. Isolated yields for the cyclic products were considerably lower due to intermolecular disulfide bond formation resulting in polymeric side products, which are separated by HPLC purification. All Gd complexes were characterized using LC ESI-MS.

Kinetic inertness

The development of new contrast agents requires compounds with high thermodynamic stability and kinetic inertness with respect to Gd dechelation. Tei et al. showed that a GdDOTA-monopropanamide derivative had a very high stability constant, $\log K_{ML} = 20.2$,³⁷ and we expected that our system with the same donor set would exhibit similar thermodynamic stability. To address kinetic inertness, we measured the full transchelation of Gd(III) from the complexes GdL1, Gd2L2 Gd3L3 to a DTPA derivative with higher thermodynamic stability. Each of these complexes was challenged with one equivalent of the ligand of MS-325 (MS-325-L) on a per gadolinium basis (e.g. Gd3L3 was challenged with 3 equivalents of MS-325-L). MS-325-L is a DTPA derivative with a biphenyl moiety that enables easy separation and monitoring of the free ligand from the MS-325 gadolinium complex by HPLC. Figure 5 shows the conversion of MS-325-L to MS-325 as a function of time for the three metallopeptides at pH 3 (10 mM citrate buffer) and 37 °C.⁴⁸

Transchelation was monitored using LC-MS, via formation of the MS-325 complex. For comparison, we also measured transchelation from the approved contrast agents [Gd(HP-DO3A)(H₂O)] (ProHance, gadoteridol) and [Gd(DTPA)(H₂O)]²⁻ (Magnevist, gadopentetate). Although thermodynamically favored, it is apparent from Figure 5 that transchelation takes place over days even at pH 3. We estimated half-times for these transchelations (time to 50% of the equilibrium value). For the approved contrast agent [Gd(DTPA)(H₂O)]²⁻, the half-time was 25 minutes. On the other hand, the metallopeptides were much more inert with half-time in the 2 – 3 day range (Table 2). Transchelation was slowest for the trimer, followed by the dimer. The approved macrocyclic agent [Gd(HP-DO3A)(H₂O)] showed even slower transchelation kinetics. These results allowed us to conclude that multimers based on Gd(DOTA) are also suitable for *in vivo* applications due to satisfactory kinetic inertness in comparison with clinically utilized Gd based agents. We were also able to confirm that multimerization has no detrimental effect on decomplexation of the metal complex, rather it appears to have a stabilizing effect.

RELAXIVITY

Per Gd relaxivities were determined by measuring T_1 at 37 °C using 20, 60, 200, 400 and 500 MHz spectrometers. Relaxivities for MS-325 (with and without the presence of HSA) as a reference compound with a long τ_R and [Gd(HP-DO3A)(H₂O)] as a reference for very short τ_R were also measured, and all the relaxivity data is tabulated in Table 3, together with results obtained from literature for the compounds with similar estimated τ_R .

At low fields such as 0.47 and 1.4T, the compounds with the highest rotational correlation times (MS-325/HSA and the trimers) exhibit the highest relaxivity. Additional rigidity through cyclization seems to provide only minor relaxivity increase for the dimeric and trimeric systems (Gd2C2 and Gd3C3). At intermediate field (4.7 T), only a moderate decrease in relaxivity is observed for the metallopeptides. In comparison, HSA-associated MS-325 exhibits a peak molecular relaxivity of above 40 mM⁻¹s⁻¹,⁴⁹ followed by rapid decrease in relaxivity upon increase of the magnetic field. Because we use the rigid GdDOTA amino acid for multimerization, both the per Gd relaxivity and per molecule relaxivity increase with increased molecular size. Figure 6a illustrates this effect where we plot the field dependent molecular relaxivity of GdL3 along with that of the approved contrast agents [Gd(HP-DO3A)(H₂O)] in PBS and MS-325 in the presence of excess HSA. At 0.47 T the molecular relaxivities of GdL3 is similar to MS-325 in HSA solution. As the field is increased the molecular relaxivity of GdL3, with its intermediate rotational correlation time, becomes higher than that of MS-325/HSA: 50% higher at 1.4 T and 350 – 450% higher at fields from 4.7 to 11.7 T. The molecular relaxivity of GdL3 is 5- to 11-fold higher than that of [Gd(HP-DO3A)(H₂O)] at all fields measured. On a per Gd basis, the

relaxivity of **GdL3** is 50 – 220% higher than either HSA-bound MS-325 or [Gd(HP-DO3A)(H₂O)] at high fields (4.7 – 11.7 T), Figure 6B.

In order to further illustrate this, we imaged a series of phantoms at 4.7 T (Figure 7). Water is used as a reference for background, [Gd(HP-DO3A)(H₂O)] as an example of a compound with a short τ_R , and MS-325 bound to HSA as an example of a complex with a long τ_R . **Gd₃L3** is shown at two different concentrations: either equimolar on a per Gd basis, or on a per molecule basis. It is evident, that **Gd₃L3** provides better contrast at this field strength than either FDA approved compound, highlighting superiority in performance of our compound with intermediate τ_R at fields above 1.5 T. Under these conditions, the signal intensity of **Gd₃L3** at equimolar Gd(III) ion concentrations was 65 % greater than [Gd(HP-DO3A)(H₂O)] and 55 % greater than MS-325/HSA. On a per molecule basis, the **Gd₃L3** phantom was 190% and 170% brighter than Gd(HP-DO3A) and MS-325/HSA, respectively.

At 9.4T, the per Gd relaxivities were 6.1 and 6.6 mM⁻¹s⁻¹ for the trimeric metalloptides. These values are also found to be higher than the relaxivities measured at 9.4T for previously reported trimeric compounds of similar composition and hydration number.^{5, 30} For compounds based on q=2 complexes, higher relaxivities can be obtained.¹⁷

Investigation of the effect of tertiary structure on relaxivity, was done by examination of the effect of disulfide bond reduction on T₁ at 0.47 and 1.41T. T₁ was measured for each sample at 37 °C and then the samples were incubated with 20 eq. TCEP for 30 minutes at room temperature to reduce the intramolecular disulfide bond and give the linear peptide. Subsequently, the T₁ values were remeasured and concentrations re-determined in order to calculate relaxivities. A slight decrease in relaxivity (7 – 14%) was observed for **Gd₂C2-red** (9 mM⁻¹s⁻¹) and **Gd₃C3-red** (11.5 mM⁻¹s⁻¹). Over all, reduction of the disulfide bond has only a slight effect on relaxivity. We hypothesize that the large Gd-chelate side chain and the Gd(III) coordination by the amide carbonyl imposes defined structure to the peptide that dominates the over-all molecule structures for both the linear and the cyclic multimers. Introduction of a secondary structure modification such as the cyclization has only a marginal influence on the relaxivity. Nevertheless, facile introduction of the disulfide bridge by use of standard peptide synthesis methodology demonstrates the modularity of our system.

The high field relaxivities that we have obtained are consistent with an intermediate rotational correlation time. By assuming that the contributions of second-sphere and outer-sphere water can be estimated from a related q=0 complex,⁵⁰ we estimate τ_R of these metalloptides to be in 150 – 600 ps range, based on the magnitude and field dependence of their relaxivities. More precise estimates of τ_R could be obtained by additional relaxation measurements using high resolution NMR with other Ln surrogates of Gd.⁵¹ Compared to the other multimers reported in Table 3, our relaxivities are similar. For a specific field strength the rotational dynamics will dictate the optimal relaxivity. The modular amino acid approach presented here offers the possibility to tune such a high field relaxivity by systematically controlling the size and nuclearity of the complex.

CONCLUSIONS

In conclusion, we were able to synthesize a single amino acid Gd chelate, Gd(DOTAla), suitable for solid phase peptide synthesis. The chelate is unique as it provides rigid and stable attachment of the metal complex to the rest of the molecule by using the amidocarbonyl of the corresponding peptide backbone as a point of attachment. Gd(DOTAla) when incorporated into a peptide exhibits one inner-sphere water ligand that has an optimal rate of water exchange for relaxometric purposes. The macrocyclic structure

of the chelate provides high thermodynamic stability and kinetic inertness with respect to transchelation or Gd dissociation. The rigid incorporation of Gd(DOTA) into a peptide scaffold allows design of contrast agents with defined rotational dynamics. Here, we described 6 new compounds containing 1 – 3 Gd(DOTA) per peptide in a linear or cyclic peptide framework. By careful control of the rotational dynamics, it is possible to design contrast agents with high relaxivities at both low and high magnetic fields. These new contrast agents were superior to commercial contrast agents [Gd(HP-DO3A)(H₂O)] and MS-325/HSA at high fields. The modularity of design, the ease of solid phase synthesis, high kinetic inertness, and optimal water exchange rate renders the Gd(DOTA) scaffold a suitable platform for the development of high field T₁ agents based on Gd.

EXPERIMENTAL SECTION

General Methods and Materials

¹H and ¹³C NMR spectra were recorded on a Varian 11.7 T NMR system equipped with a 5 mm broadband probe. Purification via HPLC of intermediates toward Fmoc-DOTA was performed using method A: Injection of crude mixture onto preparative HPLC on a Rainin, Dynamax (column: 250 mm Polaris C18) by using A: 0.1% TFA in water, B: 0.1% TFA in MeCN, flow-rate 15 mL/min, from 5% B to 95 % B over 20 min. Purification of Gd complexes was performed using method B: Injection of crude mixture onto analytical column (Phenomenex Luna, C18(2) 100/2 mm) using A: water, B: MeCN, flow-rate 0.8 mL/min, 15 min gradient from 2% B to 60 % B over 15 min. Monitoring of UV absorption was done at 220 nm. HPLC purity analysis (both UV and MS detection) was carried out on an Agilent 1100 system (column: Phenomenex Luna, C18(2) 100/2 mm) with UV detection at 220, 254 and 280 nm by using a method C: A gradient of 95 % A (0.1 % formic acid in water) to 95% B (0.1% formic acid in MeCN), flow-rate 0.8 mL/min, 1 over 15 min. Kinetic inertness measurements were also carried out using the LCMS agilent system, using method D: A gradient of 95 % A (ammonium formate, 20 mM, pH 6.8) with 5% (9:1 MeCN/20 mM ammonium formate) to 95% B (9:1 MeCN/20 mM ammonium formate), flow-rate 0.8 mL/min, 1 over 15 min.

The synthesis of ligands was carried out as shown in Schemes 1 and 2. Chemicals were supplied by Aldrich Chemical Co., Inc., and were used without further purification. Solvents (HPLC grade) were purchased from various commercial suppliers and used as received.

Luminescence

Measurements were collected by using the confocal portion of a custom-designed multimodal microscope.^{42, 43} Briefly, a continuous-wave diode laser (λ=532 nm, B&W Tek) provided excitation light that was temporally gated by an electro-optical modulator (ConOptics, Danbury, CT) with extinction ratio of approximately 200 at 532 nm. The excitation beam passed through several conditioning optics, including a beam expander with pinhole spatial filter, polarizer, shutter, dichroic mirror, scan lens, and tube lens and a 20× magnification objective lens (XLumPlan FL, Olympus, NA=0.95). With the use of a customized control software and galvanometric scanners (Cambridge Technology, Inc. Lexington, MA) the excitation beam was guided to selected locations in the approximately 600 μm field of view. The emitted luminescence was descanned and collected by using an avalanche photodiode photon counting module (APD, SPCM-AQRH-10, Perkin-Elmer, Waltham, MA) sampled at 50 MHz with a high-speed DIO card (National Instruments, Austin, TX). Data were processed by using custom-written software in C and MATLAB (Mathworks, Natick, MA). Detected luminescent photons were binned into 50 ms long bins, to yield time-dependent phosphorescence decay profiles. With the use of a nonlinear least

squares fitting routine, the resulting time-courses were fit with a single-exponential function. A sample's luminescence lifetime is equal to its fitted profile's calculated time constant.

(R)-tri-*tert*-butyl 2,2',2''-(10-(3-(benzyloxy)-2-(((benzyloxy) carbonyl)amino)-3-oxopropyl)-1,4,7,10tetraazacyclododecane-1,4,7-triyl)triacetate (4)—Cyclen (1.52g, 8.8 mmol) was dissolved in MeCN (50 mL). K₂CO₃ (1eq., 0.61g) was added and the reaction mixture was preheated to 50 °C. (*R*)-benzyl 2-(((benzyloxy)carbonyl)amino)-3-((methylsulfonyl)oxy) propanoate (2, 1.8g, 4.4 mmol) was dissolved in MeCN (20 mL) and added dropwise to the preheated solution. After 16h, the precipitate was removed by filtration and the solvent evaporated. The residue was taken up in EtOAc and extracted twice with H₂O (80 mL), and once with brine (80 mL). The organic fraction was dried with Na₂SO₄ and the solvent was evaporated *in vacuo* to afford the crude mono-cyclen derivative (1.48g, 3mmol), which was resuspended in dry MeCN (50 mL) together with K₂CO₃ (10 eq., 4.24g). *tert*-butyl bromoacetate (3.2 eq., 1.45 mL, 1.91g) was added dropwise and the mixture was stirred for 16h at room temperature. The solvent was then removed and the residue was resuspended in EtOAc and extracted with H₂O and brine. The organic fraction was collected, dried with Na₂SO₄ and the solvent was evaporated *in vacuo* to afford the crude product which was purified using preparative HPLC, method A. Yield: 1.03g (1.24 mmol, 28%). ¹H NMR (CDCl₃, 500 MHz, 298 K): δ = 7.31 – 7.30 (m, Bn-H, 10H), 5.14 – 5.04 (m, CH₂-Bn, 4H), 4.75 (brs, α-CH, 1H), 3.75 – 3.05 (m, cyclen-H/ N-CH₂-COO^tBu, 24H), 1.47 – 1.42 (m, CH₃, 27H); ¹³C NMR (CDCl₃, 125 MHz, 303 K): δ = 167.8, 167.7, 160.9, 160.7, 136.2, 134.8, 128.6, 128.5, 128.2, 127.9, 119.5, 117.2, 114.9, 112.6, 83.3, 68.0, 67.2, 55.0, 54.7, 50.9, 50.15, 27.9; LC/MS (ESI⁺): C₄₄H₆₇N₅O₁₀ m/z: calcd. 826.5 [MH⁺]; found: 826.4 (MH⁺).

(R)-2-amino-3-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)propanoic acid (5)—Compound 4 (5.5g, 6.7 mmol) was dissolved in EtOH (600 mL). Pd/C (2.9g, 10 % w/w) was added to afford a slurry which was subjected to H₂ (35 psi) for 3h. The Pd/C was filtered off and the filtrate was reduced *in vacuo* to afford the product (3.85g, 6.4 mmol) as a colorless oil which was used without further purification in the subsequent reaction step. ¹H NMR (CD₃OD, 500 MHz, 298 K): δ = 4.18 (brs, α-CH, 1H), 4.85 – 3.15 (m, cyclen-H/ N-CH₂-COO^tBu, 24H), 1.53 – 1.50 (m, CH₃, 27H); ¹³C NMR (CD₃OD, 125 MHz, 303 K): δ = 170.4, 161.4, 161.2, 83.4, 54.3, 50.1, 49.1, 26.9; LC/MS (ESI⁺): C₂₉H₅₅N₅O₈ m/z: calcd. 602.4 [MH⁺]; found: 602.5 (MH⁺).

(R)-2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)propanoic acid (6)—Compound 5 (2.395g, 3.98 mmol) was dissolved in dioxane (60 mL). Na₂CO₃ (1.27g, 11.9 mmol, 3 eq) was dissolved in H₂O. The two solutions were mixed and cooled to 0 °C. Fmoc-Cl (1.125g, 4.3 mmol) was dissolved in dioxane (5 mL) and added to the reaction mixture. The solution was allowed to warm to room temperature and stirred for 4 h. The solvent was then removed and the solid residue was dissolved in MeCN. The residual solid was filtered off and the filtrate was purified using preparative HPLC, method A. The product fractions were pooled and the solvent was removed *in vacuo* to afford the clean product as a white solid (1.81g, 2.2 mmol, 55%). ¹H NMR (CDCl₃, 500 MHz, 298 K): δ = 7.30 – 7.26 (m, FmocAr-H, 10H), 4.76 (brs, α-CH, 1H), 4.30 (m, CH₂-Fmoc, 2H), 4.15 (q, CH-Fmoc, 1H), 3.75 – 3.05 (m, cyclen-H/ N-CH₂-COO^tBu, 24H), 1.45 – 1.36 (m, CH₃, 27H); ¹³C NMR (CDCl₃, 125 MHz, 303 K): δ = 171.1, 169.7, 143.6, 141.2, 127.8, 127.2, 125.2, 119.9, 84.8, 83.3, 67.5, 54.0, 50.7, 48.3, 46.8, 27.8; LC/MS (ESI⁺): C₄₄H₆₅N₅O₁₀ m/z: calcd. 824.5 [MH⁺]; found: 824.4 (MH⁺).

Solid-phase Peptide Synthesis—Solid-phase peptide synthesis was carried out manually following standard Fmoc protocols using PEGA Rink amide resin. All peptide sequences were derived from one solid support 0.33 mmol scale using single step couplings of four equivalents of Fmoc-amino acids, two equivalents coupling agent (HATU) and 3 equivalents N-methylmorpholine (NMM) in DMF at RT (refer to scheme 2). Coupling with commercial amino acids was completed within 12h (Step i), while Fmoc-DOTAla was only used in a 1.5 equivalent excess and allowed to react with the free N terminus of the peptide for 48h (Step ii). The coupling step was followed by rinsing with DMF and deprotection with 20% piperidine in DMF for 2h. After subsequent thorough rinsing with DMF and dichloromethane, a small aliquot of solid support was removed from the batch and deprotected using cleavage cocktail (TFA: DDT: TIPS: Water (9.5:0.25:0.25:0.25)) room temperature for 2 h. The resin was filtered off and the filtrate concentrated with a gentle nitrogen flow. The intermediate was precipitated with cold diethyl ether, collected and characterized by ESI-MS. If coupling was found to be complete, the next coupling step was initiated on the main peptide batch. Once a sequence was complete, the corresponding aliquot was removed from the main resin batch and completed by addition of the terminal Fmoc-Cysteine-S-Acm. For cyclic sequences, treating the resin-bound peptide with 10 equivalents of I₂ in DMF for 6 h completed side chain deprotection with simultaneous cyclisation of the Cys residues (Step iii). After thorough rinsing of the resin with DMF and dichloromethane following the final processing step on-bead (Fmoc deprotection for linear systems, I₂ cyclisation for cyclic sequences), the crude peptide was afforded by cleaving from the resin using the acidic cleavage cocktail (see above) and isolated by cold ether precipitation, redissolution in water and lyophilization (Step iv). Because the on-bead cyclization proceeds to only approximately 60% completion, the crude peptide is further cyclized using 2% DMSO in basic H₂O (pH ~ 7.5). As epimerization occurs on the stereocenter of DOTAla, multiple peaks are detected for the corresponding diastereomers.

H₂N-C(Acm)PG-DOTAla-GC(Acm)CONH₂ (H₃L1), HPLC: R_t= 2.4/3.1 min, MS-ESI: m/z: 1043.4 (calcd. 1043.3) [M+H]⁺.

H₂N-C(Acm)PG-DOTAla-G-DOTAla-GC(Acm)CONH₂ (H₆L2), HPLC: R_t= 1.3/1.48 min, MS-ESI: m/z: 1514.6 (calcd. 1514.5) [M+H]⁺.

H₂N-C(Acm)PG-DOTAla-G-DOTAla-G-DOTAla-GC(Acm)CONH₂ (H₉L3), HPLC: R_t= 1.21/1.35 min, MS-ESI: m/z: 994.0 (calcd. 994.2) [M+2H]²⁺.

H₂N-C(S^{cycl})PG-DOTAla-GC(S^{cycl})CONH₂ (H₃C1), HPLC: R_t= 1.35/1.6 min, MS-ESI: m/z: 898.4 (calcd. 898.3) [M+H]⁺.

H₂N-C(S^{cycl})PG-DOTAla-G-DOTAla-GC(S^{cycl})CONH₂ (H₆C2), HPLC: R_t= 1.1/1.2 min, MS-ESI: m/z: 1372.5 (calcd. 1372.3) [M+H]⁺.

H₂N-C(S^{cycl})PG-DOTAla-G-DOTAla-G-DOTAla-GC(S^{cycl})CONH₂ (H₉C3), HPLC: R_t= 1.24 min, MS-ESI: m/z: 922.95 (calcd. 922.8) [M+2H]²⁺.

Gadolinium Complex Formation—Complexes were prepared by adding GdCl₃•6H₂O stock solution to a solution of ligand at pH 3 while stirring. The pH was gradually adjusted to pH 6.5 using 0.1 M NaOH solution. Complete complex formation was checked by LCMS (no residual ligand detectable). The solution was filtered and purified using preparative HPLC, method B. The Eu(III) complex is formed in analogous fashion.

H₂N-C(Acm)PG-DOTAla(Gd)-GC(Acm)CONH₂ (GdL1), HPLC: R_t= 2.9/3.3 min, MS-ESI: m/z: 1197.3 (calcd. 1197.2) [M+H]⁺.

H₂N-C(Acm)PG-DOTAla(Gd)-G-DOTAla(Gd)-GC(Acm)CONH₂ (Gd₂L₂), HPLC: R_t= 2.6/3.0 min, MS-ESI: m/z 912.5 (calcd. 912.5) [M+2H]²⁺.

H₂N-C(Acm)PG-DOTAla(Gd)-G-DOTAla(Gd)-G-DOTAla(Gd)-GC(Acm)CONH₂ (Gd₃L₃), HPLC: R_t= 3.2 min, MS-ESI: m/z: 1225.95 (calcd. 1225.8) [M+2H]²⁺.

H₂N-C(S^{cycl})PG-DOTAla(Gd)-GC(S^{cycl})CONH₂ (GdC1), HPLC: R_t= 1.13 min, MS-ESI: m/z: 1053.2 (calcd. 1053.4) [M+H]⁺.

H₂N-C(S^{cycl})PG-DOTAla(Gd)-G-DOTAla(Gd)-GC(S^{cycl})CONH₂ (Gd₂C₂), HPLC: R_t= 1.25 min, MS-ESI: m/z 840.7 (calcd. 841.5) [M+2H]²⁺.

H₂N-C(S^{cycl})PG-DOTAla(Gd)-G-DOTAla(Gd)-G-DOTAla(Gd)-GC(S^{cycl})CONH₂ (Gd₃C₃), HPLC: R_t= 1.35/1.9 min, MS-ESI: m/z: 1153.8 (calcd. 1154.6) [M+2H]²⁺.

Reduction of disulfide bond for relaxivity measurements—Complex solutions of purified, cyclic Gd complexes (concentrations of 0.1- 0.025 mM, 110 μL) in HEPES buffer (50 mM, pH 7.4) were mixed with TCEP solution (20 mM in HEPES, 10 μL) and incubated room temperature. Reduction was checked by LCMS analysis and found to be complete after 30 min.

H₂N-C(SH)PG-DOTAla(Gd)-GC(SH)CONH₂ (GdC1-red), HPLC: R_t= 2.35 min, MS-ESI: m/z: 1055.2 (calcd. 1055.2) [M+H]⁺.

H₂N-C(SH)PG-DOTAla(Gd)-G-DOTAla(Gd)-GC(SH)CONH₂ (Gd₂C₂-red), HPLC: R_t= 2.8-3.1 min, MS-ESI: m/z 841.7 (calcd. 842.0) [M+2H]²⁺.

H₂N-C(SH)PG-DOTAla(Gd)-G-DOTAla(Gd)-G-DOTAla(Gd)-GC(SH)CONH₂ (Gd₃C₃-red), HPLC: R_t= 3.1-3.3 min, MS-ESI: m/z: 1155 (calcd. 1155) [M+2H]²⁺.

Measurement of kinetic inertness—Stock solutions of MS-325-L and GdL1, Gd₂L₂ and Gd₃L₃ were prepared in 50 mM citrate buffer at pH 3.0. MS-325-L was added to solutions of the Gd complexes and incubated at 37 °C. The final concentrations of the metal complexes were 0.1 mM, while the concentration of MS-325-L was adjusted according to the amounts of Gd complexes per metalloprotein present. A 10 μL aliquot was removed for HPLC analysis and analyzed while the remainder of the solution was incubated at 37 °C. A 10 μL aliquot was removed and analyzed at 5, 10, 25, 46, 78, 96, 122, 141 and 244h. As a reference, Gd(HP-DO3A) was subjected to MS-325-L under same conditions and measured at time points 0.3, 1.5, 4, 6, 8, 24, 168 and 336h.

Measurement of relaxivity—Longitudinal relaxation times T₁, were measured on Bruker Minispecs mq20 (0.47T) and mq60 (1.41T), a Bruker Bioscan horizontal bore 4.7T, 9.4T and 11.7T Varian NMR spectrometers. T₁ was measured by using an inversion recovery method with 10 inversion time values ranging from 0.05 × T₁ to 5 × T₁. Relaxivity was calculated from a linear plot of 3 or 4 different concentrations (ranging from 0.01 to 0.5 mM, depending on amount of compound isolated) versus the corresponding inverse relaxation times. All samples were measured at 37 °C using either the internal temperature control of the instrument (0.47, 1.41, 9.4 and 11.7 T) or a warm air blower (4.7 T). MS-325/HSA was prepared in a 4.5% w/v solution of HSA (0.66 mM) in PBS. The MS-325 concentration (in presence of HSA) ranged from 0.05 to 0.15 mM.

¹⁷O NMR of GdL1 solution for determination of τ_M—¹⁷O NMR measurements of solutions were performed at 11.7 T on 150 μL samples contained in 2-mm-shigemi tubes

inside a 5 mm standard NMR tube on a Varian spectrometer. Temperature was regulated by air flow controlled by a Varian VT unit. ^{17}O transverse relaxation times of distilled water (pH 3) containing 5 % enriched $^{17}\text{OH}_2$ or a 6.88 mM solution of GdL1 (pH 7.4, 50 mM HEPES buffer) were measured using a CPMG sequence. The concentration of the sample was determined by ICP-MS. Reduced relaxation rates, $1/T_{2r}$ were calculated from the difference of $1/T_2$ between the GdL1 sample and the water blank, and then divided by the mole fraction of coordinated water. The temperature dependence of $1/T_{2r}$ was fit to a 4-parameter model as previously described.²² The Gd-O hyperfine coupling constant, $A/\hbar$, was assumed to be 3.8×10^6 rad/s,⁴⁵ the Gd-O distance was assumed to be 3.1 Å.⁵²

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Figure 1. Molecular parameters that influence relaxivity: rotation (τ_R), water exchange (τ_M), hydration number (q) and electronic relaxation (T_{1e}).

Figure 2.

Top row: Schematic depiction of previously explored Gd complexes with τ_R between 0.35 and 1 ns (A, B, C), as well as the novel approach described here (D). Bottom row: Examples for molecules reported using approaches A,¹⁷ B⁵ and C.¹⁸

Figure 3.

Top: Structures of approved Gd-based agents $[\text{Gd}(\text{DOTA})(\text{H}_2\text{O})]^-$ and $[\text{Gd}(\text{HP-DO3A})(\text{H}_2\text{O})]$. Bottom: various approaches to conjugated DOTA derivatives. E and F show previously investigated Gd(DOTA) type complexes with optimal water exchange properties. G represents a previously explored lysine derivative of DOTA without the dual attachment strategy. Compound H represents our approach using dual attachment to the peptide to limit internal motion of the complex.

Figure 4. Temperature dependence of the ^{17}O NMR (11.7T) reduced transverse relaxation rates of GdL1 (6.8 mM). Solid line represents fit to the data to determine the water exchange rate.

Figure 5. Kinetic inertness of Gd(DOTA) derivatives. Transchelation of Gd from linear complexes GdL1(●), Gd₂L2(■), Gd₃L3 (▲) and [Gd(HP-DO3A)(H₂O)] (◆) to MS-325 at pH 3, 37 °C. Data shown for the first 168 hours of the reaction.

Figure 6.

A and 6B. Relaxivities of Gd₃L3(●), MS-325 with excess HSA (▽) and [Gd(HP-DO3A)(H₂O)] (◆) as a function of magnetic field at 37 °C. (A) Relaxivity plotted per [molecule] showing that Gd₃L3 with its intermediate correlation time is a much more potent relaxation agent than slow or fast tumbling compounds at 60 MHz and higher frequencies. (B) Relaxivity plotted per [Gd] shows that the intermediate correlation time of Gd₃L3 results in higher relaxivities at high fields.

Figure 7.

Gradient echo MR image acquired at 4.7T ($T_E = 6$ ms and $T_R = 30$ ms, flip angle = 90°) of equimolar solutions of [Gd(HP-DO₃A)(H₂O)], MS-325 in HSA (0.66 mM), Gd₃L₃ in H₂O at both equal Gd(III) ionic concentration and equal molecular concentration.

Scheme 1.
Synthesis of compound **6**.

Scheme 2.

Synthesis of new contrast agents described in this paper. (i) 1.) 4 eq. Fmoc-X (X= Gly, Phe, Cys(Acm)), 4eq. HATU, 4.5 eq. NMM, DMF, 12h 2.) 20% piperidine in DMF, 2h (ii) 1.) 1.5 eq. **6**, 2 eq. HATU, 3 eq. NMM, 2.) 20% piperidine in DMF (iii) 10 eq. I₂, DMF, 6h (iv) TFA: DDT: TIPS: Water (9.5:0.25:0.25:0.25), 6h (v) DMSO (2% v/v), H₂O (pH 8), 12h.

Table 1

Water exchange kinetic parameters for GdL1 and comparison with the Gd(III) complexes of propionamide and propionate derivatives E and F (Figure 3). $^{310}\text{T}_{1e} = 160 \pm 124$ ns and $\Delta E^\ddagger = 21 \pm 18$ kJ mol⁻¹ for GdL1.

Gd complex	E ³⁷	F ³⁸	GdL1
$^{310}k_{ex} \times 10^6$ (s ⁻¹)	84	42	60 ± 6
$\Delta H^\ddagger$ (kJ mol ⁻¹)	34.0	19.1	41.7 ± 3.2
$^{310}\tau_M$ (ns)	12	24	17 ± 2

Table 2

Half-times for Gd transchelation to MS-325 at pH 3, 37 °C, with a Gd : MS-325-L ratio of 1:1 at 0.1 mM Gd complex concentration.

Gd complex	$t_{1/2}$ [h]
GdL1	39±3
Gd ₂ L2	52±3
Gd ₃ L3	61±4
Gd(DO3A-HP)	91±6
Gd(DTPA)	0.42±0.18

Table 3

Measured per Gd relaxivities as a function of proton Larmor frequency at 37 °C for the linear and cyclic systems described in this work along with Gd(HP-DO3A)(H₂O)] and MS-325 measured in the presence and absence of 4.5% human serum albumin (HSA). For comparison literature data with examples of compounds **A** (*q* = 2),¹⁷ **B**,⁵ **C** (data obtained at 25 °C)¹⁸ are included.

	Relaxivity [mM ⁻¹ s ⁻¹] per Gd				
	20 MHz	60 MHz	200 MHz	400 MHz	500 MHz
GdL1	8.1	7.4	7	5.8	4.9
Gd ₂ L2	10.8	9.9	8.3	6.1	4.9
Gd ₃ L3	13.2	12.2	9	6.1	4.7
GdC1	8.3	7.1	7.3	5.1	4.5
Gd ₂ C2	11.4	10.6	7.5	5.7	4.5
Gd ₃ C3	12.7	12.3	9.2	6.6	5.5
[Gd(HP-DO3A)]	4.3	3.2	3.6	3.0	2.9
MS-325	6.8 ^d	5.4 ^d	5.7	4.8	4.7
MS-325 w HSA	42 ^d	23.8 ^d	5.0	4.1	3.7
{Fe[Gd ₂ bpy(DTTA) ₂ (H ₂ O) ₄] ₃ } ⁴⁻	20.1 ^a	26.8 ^a	15.9 ^a	8.3 ^a	n.a.
[Bm(Gd(HPN3DO3A)(H ₂ O)) ₃]	~ 15 ^b	15.4 ^b	n.a.	4.8 ^b	n.a.
[Gd(gDOTA-Glu12)(H ₂ O)] ⁻	23.5 ^c	~ 25 ^c	n.a.	n.a.	n.a.

All data this work except

^aRef.¹⁷;

^bRef.⁵;

^cRef.¹⁸;

^ddata at 20 and 60 MHz from ref²²;

n.a. ; data not available.