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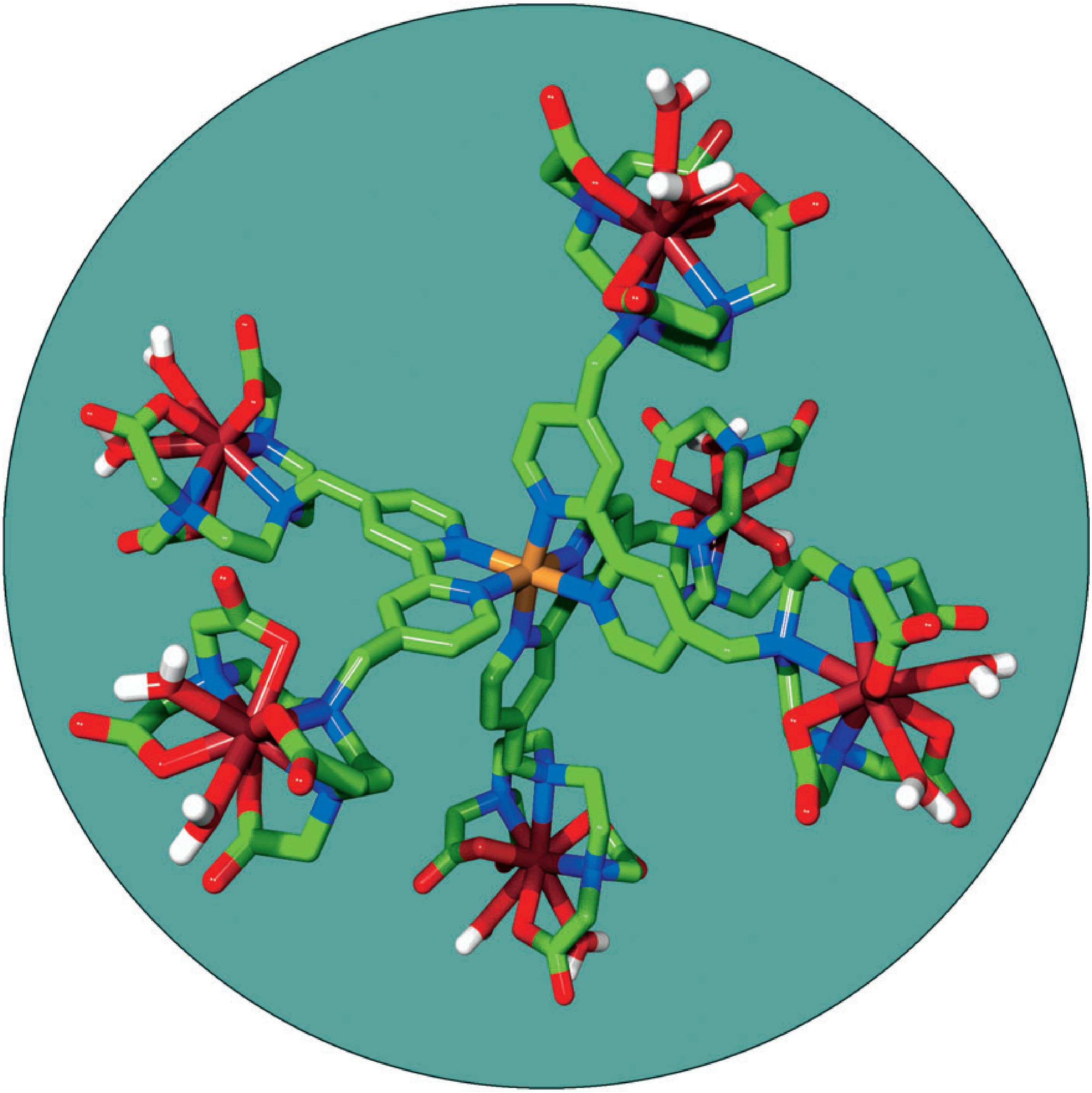

Ein Poly(aminocarboxylat)-Bipyridin-Ligand L bildet mit Fe^{II}- und Gd^{III}-Ionen die stabile sternförmige Struktur $[Fe\{GdL(H_2O)_2\}_3]^{4-}$, die über eine bemerkenswerte Relaxivität verfügt. Auf den folgenden Seiten diskutieren E. Tóth et al. die Eigenschaften dieses Komplexes hinsichtlich einer möglichen Anwendung als Kontrastmittel in der Kernspintomographie.

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MRI Contrast Agents

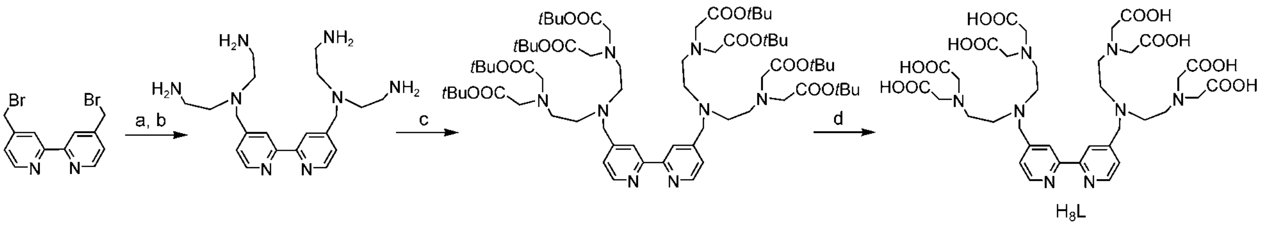
High Relaxivity Confined to a Small Molecular Space: A Metallostar-Based, Potential MRI Contrast Agent**

João Bruno Livramento, Éva Tóth, Angélique Sour, Alain Borel, André E. Merbach, and Robert Ruloff*

The 2003 Nobel Prize in medicine was awarded for discoveries concerning magnetic resonance imaging (MRI), a technique that has revolutionalized medical diagnostics over the last two decades.[1] Such spectacular developments would not have been possible without the use of paramagnetic contrast agents, the majority of which comprise Gd^{III} complexes.[2] By reducing the relaxation time of the surrounding water protons, these agents enhance the intrinsic contrast of magnetic resonance images. The efficacy of a contrast agent is expressed by its relaxivity, r_1, which is the enhancement of the longitudinal proton relaxation rate induced by the paramagnetic agent, referred to a concentration of Gd^{3+} of 1 mmol L^{-1} (molar relaxivity per Gd). Theory predicts high relaxivity for a Gd^{III} complex when its rotation and electron spin relaxation are slow and when the rate of water exchange between the inner sphere and the bulk solvent is optimal. Novel applications in MRI call for very highly efficient contrast agents. It implies not only high molar relaxivity per Gd^{III} ion but high relaxivity per molecular volume/mass. For example, in cell imaging, biological constraints limit the amount of contrast agent that can be delivered into one cell without destroying it. Consequently, agents with many efficiently relaxing paramagnetic centers confined into a small space are advantageous over large macromolecules with few Gd^{III} centers.

Recently we started to exploit heterometallic assemblies in the aim of confining high relaxivity into a small molecular space. We reported a terpyridine (tpy) ligand functionalized with a poly(aminocarboxylate) group DTTA (diethylenetriamine-*N,N,N″,N″*-tetraacetate), (tpy-DTTA)$^{4-}$, which has distinct binding sites for Fe^{II} and Gd^{III} ions.[3] In aqueous solution and in the presence of these metal ions, the ligand undergoes self-assembly to form a rigid supramolecular structure, [Fe^{II}(tpy-DTTA)$_2$Gd$_2$]. Prior to our work, Desreux and co-workers reported a [Gd(DO3A)(phen)] derivative (DO3A = 1,4,7-tri(carboxymethyl)-1,4,7,10-tetraazacyclododecane, phen = 1,10-phenanthroline) that self-assembles with Fe^{II} ions to yield a tetranuclear FeGd$_3$ entity.[4]

Here we report a novel heterotritopic ligand, H$_8$L, which comprises a 2,2′-bipyridine moiety for specific binding to Fe^{II} ions and two poly(aminocarboxylate) groups for binding to Gd^{III} ions (Scheme 1). The ligand self-assembles with Fe^{II} and Gd^{III} into a metallostar [Fe{Gd$_2$L(H$_2$O)$_4$}$_3$]$^{4-}$ structure. The rational design of H$_8$L consists of several key features: 1) The appropriate choice of the poly(aminocarboxylate) group, which has the same chelating unit as the previously described TTAHA (*N*-tris(2-aminoethyl)amine-*N′,N′,N″,N″,N‴,N‴*-hexaacetate) ligand,[5] ensures sufficient thermodynamic stability for the Gd^{III} complex, an important factor for in vivo safety. It also guarantees faster water exchange than that for commercial agents, thus closer to optimal. The Gd^{III} complex has two inner-sphere water molecules to double the inner-sphere contribution to the relaxivity. Indeed, preliminary ^{17}O NMR spectroscopy experiments on the metallostar confirmed the presence of two inner-sphere water molecules and point to a water-exchange rate which is close to that previously published for [Gd(TTAHA)(H$_2$O)$_2$]$^{3-}$.[5] A complete, variable-temperature ^{17}O NMR spectroscopic study is in progress and will be reported in due course. 2) Fe^{II} ions can accommodate three strongly coordinating 2,2′-bipyridine units which increases the number of Gd^{III} centers on one iron core to six. The accumulation of six Gd^{III} ions in a small space represents a clear advantage over [Fe^{II}(tpy-DTTA)$_2$Gd$_2$]. The negative charge of [Fe{Gd$_2$L(H$_2$O)$_4$}$_3$]$^{4-}$ is also favorable for high solubility in water. 3) The linking between the Fe^{II} and Gd^{III} binding sites is designed to minimize internal flexibility that could reduce the relaxivity gained by the increased molecular size. All of these features contribute to a high relaxivity per Gd^{III} center which, for the metallostar with six Gd^{III} centers, should sum up to a remarkable relaxivity confined to a small space.



Scheme 1. Reagents and conditions: a) protected triamine, K$_2$CO$_3$, acetonitrile; b) HCl (6 M), followed by flash chromatography; c) *tert*-butyl bromoacetate, DIEA, KI, DMF; d) HCl (6 M), followed by ion-exchange chromatography. DIEA = *N,N′*-diisopropylethylamine, DMF = *N,N*-dimethylformamide.

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The initial step in the synthesis of the ligand is the nucleophilic substitution of 4,4′-bis(bromomethyl)-2,2′-bipyridine with two protected diethylenetriamine units (Scheme 1). The following carboxymethylation, ester hydrolysis, and ion-exchange chromatography steps are all straightforward. The ligand, which was obtained as a highly water-soluble and partially protonated ammonium salt ($(H_{4.5}(NH_4)_{3.5}L)$), was characterized by ^{1}H NMR spectroscopy, ESI-MS, and elemental analysis.[6] The $[Gd_2L(H_2O)_4]^{2-}$ complex is prepared by mixing the ligand and Gd^{3+} ions in a 1:2 molar ratio at pH 6. The metallostar $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ (Figure 1) forms instantaneously upon addition of Fe^{2+} to a solution of $[Gd_2L(H_2O)_4]^{2-}$ (convergent approach), and the formation of the complex is indicated by the appearance of an intense red color.

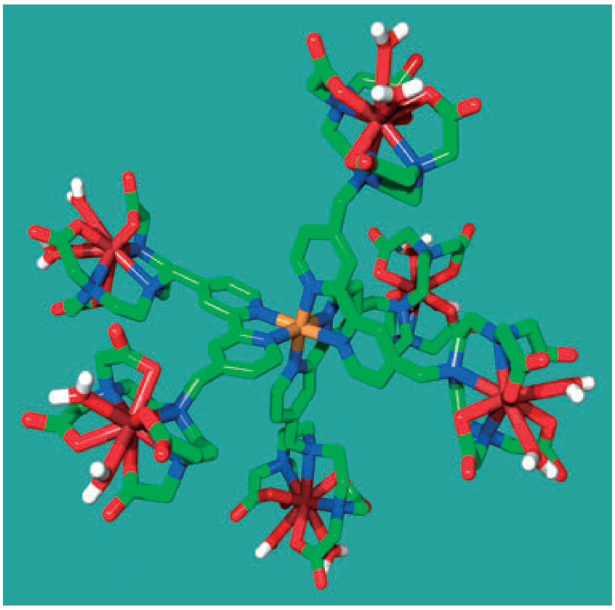

Figure 1. Framework molecular model of $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$. Hydrogen atoms have been removed for clarity. The Gd^{III} chelates, which are bound through a short spacer attached to the *para* position of the pyridyl moieties, form an octahedron around the central Fe^{II} ion that essentially reproduces the polyhedron of the bipyridine nitrogen atoms.

The dinuclear $[Gd_2L(H_2O)_4]^{2-}$ species exhibits a fairly high relaxivity (12.5 $mM^{-1}s^{-1}$) relative to dinuclear complexes such as $[pip\{Gd(DO3A)(H_2O)\}_2]$ (pip = piperidine) or $[bisoxa\{Gd(DO3A)(H_2O)\}_2]$ (5.58 and 4.43 $mM^{-1}s^{-1}$, respectively; 40 MHz, 37 °C).[7] This difference is explained by the two inner-sphere water molecules and their faster exchange, as well as the limited internal flexibility of $[Gd_2L(H_2O)_4]^{2-}$. The formation of the large and rigid $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ metallostar from $[Gd_2L(H_2O)_4]^{2-}$ increases the rotational correlation time, τ_R, whereas the water-exchange rate remains unchanged. The longer τ_R value results in a substantial relaxivity gain. Consequently, the formation of the metallostar can be followed by relaxometry. Upon adding increasing amounts of Fe^{II} ions to a solution of $[Gd_2L(H_2O)_4]^{2-}$, the relaxivity continuously increases until a molar ratio of $Fe^{II}/[Gd_2L(H_2O)_4]^{2-}$ of 1:3, after which it remains constant (Figure 2). The sharp break in the relaxometric titration curve indicates a 1:3 stoichiometry of the metallostar as expected for an Fe^{II}–2,2′-bipyridine complex. It proves that the pending Gd^{III}–poly(aminocarboxylate) units do not sterically hinder the binding of three bipyridine units per Fe^{II} ion. Molecular modeling studies also showed that the metallostar is sterically not overcrowded (Figure 1). The invariance of the relaxivity at molar ratios of $Fe^{II}/[Gd_2L(H_2O)_4]^{2-}$ higher than 1:3 points to the exclusive formation of $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$. It also shows that Fe^{II} is not oxidized to Fe^{III} which would likely replace Gd^{III} in the poly(aminocarboxylate) and the consequent release of free Gd^{3+} would decrease the relaxivity ($r_1 = 11.2\ mM^{-1}s^{-1}$ for $Gd(H_2O)_8^{3+}$ at 30 MHz and 25 °C).

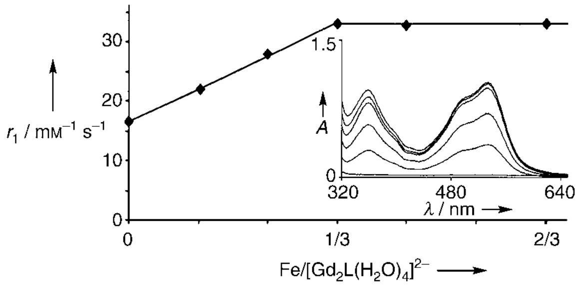

Figure 2. Relaxivity titration of $[Gd_2L(H_2O)_4]^{2-}$ with Fe^{II} at pH 6.0, 25 °C, $\nu = 30$ MHz, and $c_{Gd} = 1$ mM. Inset: UV/Vis spectra of the samples used for the relaxivity titration are shown. The absorption increases with an increasing ratio of $Fe/[Gd_2L(H_2O)_4]^{2-}$.

The formation of the metallostar was also monitored by UV/Vis spectrophotometry. Upon addition of Fe^{II} ions to a solution of $[Gd_2L(H_2O)_4]^{2-}$, new absorption bands appear at 320–640 nm. Their intensity increases until a molar ratio of 1:3 $Fe^{II}/[Gd_2L(H_2O)_4]^{2-}$, then it remains constant (the small further increase is due to the presence of free Fe^{II} ions; Figure 2).

Water proton relaxivities were measured for both the dinuclear $[Gd_2L(H_2O)_4]^{2-}$ complex and the metallostar $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ (25 °C; 0.01–600 MHz; Figure 3). Such nuclear magnetic relaxation dispersion (NMRD) profiles are widely used for the characterization of MRI contrast agents. As the consequence of slower tumbling, the relaxivities at almost all frequencies are considerably higher for the metal-

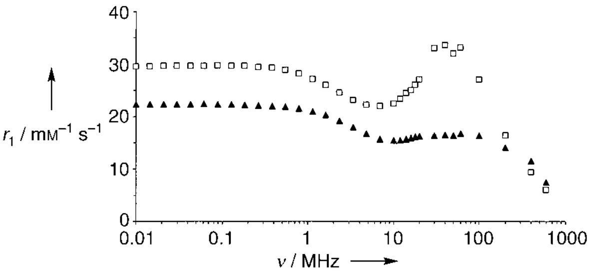

Figure 3. $1/T_1$ (T_1 = longitudinal relaxation time) NMRD profiles of solutions of $[Gd_2L(H_2O)_4]^{2-}$ (▲) and $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ (□). $c_{Gd} = 1$ mM, pH 6.0, 25 °C.

lostar than for the dinuclear complex, and a maximum increase is observed at 30–60 MHz. The profile of $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ shows the highfield peak typical of slowly rotating complexes ($r_{1\,max} = 33.6\ mM^{-1}s^{-1}$ at 40 MHz). The metallostar is rather small compared to usual macromolecular contrast agents. However, the concurrent chelation of the multifunctional ligand to Fe^{II} and Gd^{III} ions forces the rotational correlation time of the Gd^{III}–water-proton vector, which is important for relaxivity, to be close to that of the entire assembly. Thus, by strongly reducing internal flexibility, the advantage of the increased molecular size is maximized. Notably, the peak at high field is unusually broad with a relaxivity value that exceeds $16\ mM^{-1}s^{-1}$ even at 200 MHz. Contrast agents that exhibit high relaxivities over a broad frequency range and, in particular, at high frequencies are interesting as modern clinical practice tends to use high magnetic fields (> 60 MHz) to increase sensitivity.

In terms of molar relaxivity, our metallostar complex is among the best molecules reported. Its relaxivity is comparable to that of a Gd(DOTA)-loaded (DOTA = 1,4,7,10-tetra(carboxymethyl)-1,4,7,10-tetraazacyclododecane) Generation 10 dendrimer (relaxivity values of 27.0 and $33.2\ mM^{-1}s^{-1}$ at 20 and 60 MHz, respectively, at 25 °C for $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ compared to 36 and $27\ mM^{-1}s^{-1}$ at 23 °C for the G10 dendrimer).[8] This G10 dendrimer, with a molecular weight of 3000 kDa and 1860 Gd^{III} chelates on the surface, is considered to represent a high "Gd^{III} density". Such dendrimers have been designed for receptor targeting as they can deliver a concentrated relaxation effect to a given receptor binding site. Indeed, Wiener and co-workers used Gd(DTPA) (diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid) dendrimers conjugated with folic acid for visualizing the folate receptor, which is overexpressed in many tumors.[9]

The complex $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ contains six Gd^{III} ions, each exhibiting high relaxivity, for a molecular mass of $3744\ g\,mol^{-1}$. Consequently, its efficiency by a unity mass is particularly high. Rather than determine the usual molar relaxivity per Gd, r_1, which is not well-suited to characterize contrast agents in this respect, instead one can compare "effective" or "mass relaxivities", which are defined as the enhancement of the relaxation rate by a unit mass ($g\,L^{-1}$) of the contrast agent (n_{Gd} is the number of Gd ions per molecule and M_w is the molecular weight of the complex; the multiplication factor of 1000 is to obtain convenient numbers) according to Equation (1).

$$\textit{effective relaxivity} = \frac{r_1\, n_{Gd}}{M_w} \times 1000 \quad \left(\frac{mM^{-1}\,s^{-1}}{g\,mol^{-1}} \text{ or } (g/L)^{-1}\,s^{-1} \right) \qquad (1)$$

High "effective relaxivities" are required for applications such as cell imaging in which a sufficient relaxation effect has to be produced by a limited mass of the agent. Figure 4 compares "effective relaxivities" for commercial and potential contrast agents and shows that the dinuclear $[Gd_2L(H_2O)_4]^{2-}$ species and especially the metallostar $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ are remarkably powerful contrast agents in terms of efficacy by unity mass.

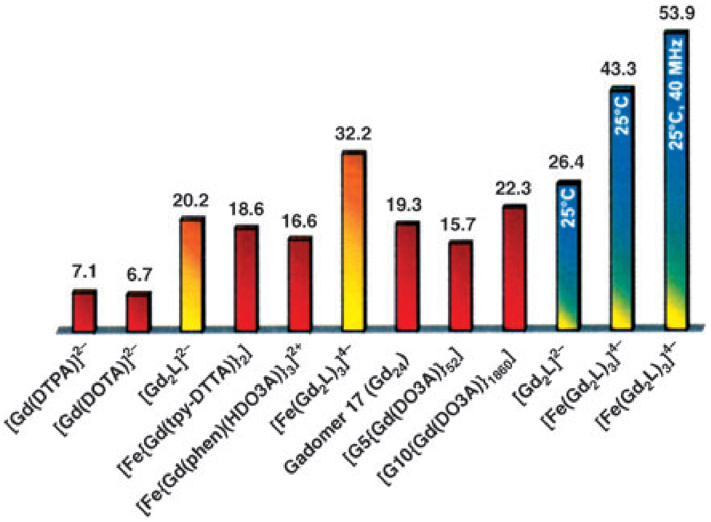

Figure 4. Comparison of the effective (mass) relaxivities (calculated according to Equation (1)) for a series of Gd^{III} complexes. The monomers $[Gd(DTPA)]^{2-}$ and $[Gd(DOTA)]^{-}$ are commercial contrast agents.[7] $[Fe\{Gd(tpy\text{-}DTTA)\}_2]$ and $[Fe\{Gd(phen)(HDO3A)\}_3]^{2+}$[4] are self-assembled structures. Gadomer 17,[10] $[G5\{Gd(DO3A)\}_{52}]$,[11] and $[G10\{Gd(DOTA)\}_{1850}]$[8] are dendrimers. The effective relaxivities were calculated at 20 MHz and 37 °C, except for the last three columns which were determined at 25 °C. ($r_1 = 20.17\ mM^{-1}s^{-1}$, the molar relaxivity per Gd for $[Fe(Gd_2L(H_2O)_4)_3]^{4-}$ at 20 MHz and 37 °C used for the calculation of the "effective relaxivity".)

Desreux and co-workers proposed the phenanthroline–Gd(HDO3A) derivative as a contrast agent responsive to Fe^{II} concentration, however, such an application did not materialize. In addition to the large increase in the relaxivity upon formation of $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ from $[Gd_2L(H_2O)_4]^{2-}$ (≈ 100 % at 30–60 MHz), our system has practical advantages over [Gd(phen)(HDO3A)]. The ligand synthesis is straightforward, and the structural diversity, as it exists for $[Fe\{Gd(phen)(HDO3A)\}_3]$, is largely reduced by the higher symmetry of L^{8-}.

In conclusion, we have reported a heterometallic, self-assembled metallostar, $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$, which exhibits a particularly high relaxivity for its moderate molecular weight. This is explained in terms of a rigid supramolecular structure and two inner-sphere water molecules with an exchange rate that lies close to optimal. The presence of six efficiently relaxing Gd^{III} centers within one metallostar leads to an exceptionally high relaxivity confined to a small molecular space (high density of relaxivity). To our knowledge this is the highest relaxivity per molecular mass ever reported for a Gd^{III} complex. The ligand L^{8-} is also a prime candidate as a terminal ligand for constructing larger-size, Fe^{II} (or Ru^{II})-based metallostars or metallodendrimers that are loaded with Gd^{III} on the surface.

Experimental Section

$[Gd_2L(H_2O)_4]^{2-}$ was prepared by adding solid L to a solution of $GdCl_3$ in 1:2 molar ratio (pH 6.0; 50 mM MES (2-morpholinoethanesulfonic acid) buffer). $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$ was prepared by mixing a solution of $[Gd_2L(H_2O)_4]^{2-}$ with a freshly prepared solution of Fe^{II} ions ($Fe(NH_4)_2(SO_4) \times 6H_2O$) in 3:1 molar ratio (pH 6.0; 5 mM MES). ESI-MS for $[Gd_2L(H_2O)_4]^{2-}$: *m*/*z* (%): 579.3 (100) [M^{2-}], 1239.2 (68) [$(MH{+}2CH_3CN)^{-}$], 1180.3 (50) [MNa^{-}], 1159.2 (23 %) [MH^{-}]; ESI-MS for $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$: *m*/*z* (%): 579.3 (100)

[$M-[\mathrm{Fe(Gd_2L)_2}]^{2-}$], 882.3 (75) [$M^{4-}$], 1184.7 (43 %) [$M\mathrm{Na}^{3-}$]. The isotopic peak patterns in both cases agree with simulated spectra. All manipulations with the metallostar were carried out with the exclusion of oxygen to avoid oxidation of $\mathrm{Fe^{II}}$. The $1/T_1$ NMRD profiles were recorded on a Stelar Spinmaster Fast Field Cycling NMR relaxometer ($B = 2.35 \times 10^{-4}$–0.47 T, proton Larmor frequencies 0.01–20 MHz), on Bruker Minispecs (30, 40, and 60 MHz), and on Bruker spectrometers (50, 100, 200, 400, and 600 MHz). The UV/Vis titration was performed on a Perkin-Elmer Lambda 19 spectrometer.

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