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Water-Soluble Gadofullerenes: Toward High-Relaxivity, pH-Responsive MRI Contrast Agents

Éva Tóth,^{*,†} Robert D. Bolskar,[‡] Alain Borel,[†] Gabriel González,^{†,§} Lothar Helm,[†] André E. Merbach,[†] Balaji Sitharaman,^{||} and Lon J. Wilson^{*,||}

Contribution from the Laboratoire de Chimie Inorganique et Bioinorganique, Ecole Polytechnique Fédérale de Lausanne, EPFL-BCH, CH-1015 Lausanne, Switzerland;

TDA Research, Inc., 12345 West 52nd Avenue, Wheat Ridge, Colorado 80033;

Departament de Química Inorgànica, Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain; and Department of Chemistry, Center for Nanoscale Science and Technology, Center for Biological and Environmental Nanotechnology, Rice University, Houston, Texas 77251-1892

Received September 2, 2004; E-mail: eva.jakabtoth@epfl.ch

Abstract: The water-soluble endohedral gadofullerene derivatives, $\text{Gd}@\text{C}_{60}(\text{OH})_x$ and $\text{Gd}@\text{C}_{60}[\text{C}(\text{COOH})_2]_{10}$, have been characterized with regard to their MRI contrast agent properties. Water-proton relaxivities have been measured in aqueous solution at variable temperature (278–335 K), and for the first time for gadofullerenes, relaxivities as a function of magnetic field (5×10^{-4} to 9.4 T; NMRD profiles) are also reported. Both compounds show relaxivity maxima at high magnetic fields (30–60 MHz) with a maximum relaxivity of $10.4 \text{ mM}^{-1} \text{ s}^{-1}$ for $\text{Gd}@\text{C}_{60}[\text{C}(\text{COOH})_2]_{10}$ and $38.5 \text{ mM}^{-1} \text{ s}^{-1}$ for $\text{Gd}@\text{C}_{60}(\text{OH})_x$ at 299 K. Variable-temperature, transverse and longitudinal ^{17}O relaxation rates, and chemical shifts have been measured at three magnetic fields ($B = 1.41, 4.7,$ and 9.4 T), and the results point exclusively to an outer sphere relaxation mechanism. The NMRD profiles have been analyzed in terms of slow rotational motion with a long rotational correlation time calculated to be $\tau_R^{298} = 2.6 \text{ ns}$. The proton exchange rate obtained for $\text{Gd}@\text{C}_{60}[\text{C}(\text{COOH})_2]_{10}$ is $k_{\text{ex}}^{298} = 1.4 \times 10^7 \text{ s}^{-1}$ which is consistent with the exchange rate previously determined for malonic acid. The proton relaxivities for both gadofullerene derivatives increase strongly with decreasing pH (pH: 3–12). This behavior results from a pH-dependent aggregation of $\text{Gd}@\text{C}_{60}(\text{OH})_x$ and $\text{Gd}@\text{C}_{60}[\text{C}(\text{COOH})_2]_{10}$, which has been characterized by dynamic light scattering measurements. The pH dependency of the proton relaxivities makes these gadofullerene derivatives prime candidates for pH-responsive MRI contrast agent applications.

Introduction

Endohedral metallofullerenes are fullerenes that encapsulate metal atom(s) within the fullerene interior space.¹ Water-soluble derivatives of endohedral metallofullerenes possess unique potential for medicinal applications, since the fullerene cage protects the encapsulated metal ion from external chemical attack and metal ion release in the body. In this respect, metallofullerenes are also especially promising for nuclear medicine and radiopharmaceuticals.^{2,3} In addition, gadolinium-containing fullerenes have been proposed as contrast enhancing agents for magnetic resonance imaging (MRI).^{4–7} Phantom and in vivo MRI studies both have shown promising results.^{5–7}

Currently, stable Gd^{3+} poly(aminocarboxylate) complexes are widely used as contrast agents in MRI.⁸ These agents are intravenously administered to patients, and by reducing the relaxation time of water protons in the effected tissues, they help produce a higher quality (higher contrast) diagnostic image. The efficacy of a contrast agent is commonly expressed by its proton relaxivity, r_1 , defined as the paramagnetic longitudinal relaxation rate enhancement of the water protons by unity concentration (mM) of the agent. Although MRI usually provides good spatial resolution, sensitivity is often a problematic issue, especially in novel applications such as the targeting of specific cells or tissues. Since the number of Gd^{3+} complexes that can be delivered or attached to a specific cell is largely limited by biological constraints, the efficacy of each Gd^{3+} -center must be improved in order to induce sufficient signal

[†] Ecole Polytechnique Fédérale de Lausanne.

[‡] TDA Research, Inc..

[§] Universitat de Barcelona.

^{||} Rice University.

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intensity to image individual cells.^{9,10} For this reason, the search for more efficient, higher-relaxivity MRI agents represents a key objective in current contrast agent development. In addition to greater efficacy, low toxicity is also an absolute requirement. For Gd³⁺ complexes, toxicity is largely related to the release of the metal ion during metabolic processes.¹¹ In this regard, the entrapment of the Gd³⁺ ion within a fullerene cage for gadofullerenes helps ensure an especially safe in vivo use of metallofullerene contrast agents, permitting applications requiring longer residency times.

Polyhydroxyl derivatives of gadofullerenes have been proposed by several groups as potential MRI contrast agents. The published longitudinal proton relaxivities for these gadofullerenols are remarkably high, while showing a large variation. Zhang et al. reported a relaxivity of $r_1 = 47 \text{ mM}^{-1} \text{ s}^{-1}$ (9.4 T),⁴ Wilson et al. found $r_1 = 20 \text{ mM}^{-1} \text{ s}^{-1}$ (0.47 T, 40 °C),² and Shinohara measured a particularly high longitudinal proton relaxivity for Gd@C₈₂(OH)₄₀ ($81 \text{ mM}^{-1} \text{ s}^{-1}$ at 25 °C, $B = 1.0 \text{ T}$).⁵ Endohedral metallofullerenols with a series of lanthanide metal ions have been also reported for M@C₈₂(OH)_n with M³⁺ = La, Ce, Gd, Dy, and Er.⁶ The metallofullerenols containing low magnetic moment ions (La³⁺ and Ce³⁺) have almost zero proton relaxivities, whereas those containing high magnetic moment ions, such as Gd³⁺, Dy³⁺, and Er³⁺, exhibit higher proton relaxivities, with Gd@C₈₂(OH)_n being the most efficient relaxing agent. For these latter cases, both longitudinal and transverse proton relaxivities are significantly higher than those for the corresponding lanthanoid-DTPA chelates. To date, the origin of these high relaxivities and the relaxation mechanism involved have not been fully elucidated.

Recently, Bolskar and Wilson et al. reported the synthesis of the first water-soluble Gd@C₆₀ derivatives.⁷ Although M@C₆₀ and related endohedral metallofullerenes represent the major portion of all metallofullerenes produced in the traditional arc synthesis, their low solubility hampered systematic investigations until procedures were developed to solubilize Gd@C₆₀ by forming the malonate derivative, Gd@C₆₀[C(COOH)₂]₁₀. Its proton relaxivity was found to be lower than those of the gadofullerenol compounds previously reported, being comparable to the relaxivity of commercially available Gd³⁺-based MRI contrast agents. Furthermore, an in vivo biodistribution study demonstrated that Gd@C₆₀[C(COOH)₂]₁₀ is the first water-soluble endohedral metallofullerene with decreased uptake by the reticuloendothelial system (RES) and facile excretion via the urinary tract. The synthetic method used to produce the water-soluble carboxylated M@C₆₀ derivatives employed direct derivatization at the solid/solution interface of a high-metallofullerene content soot. This soot material, dominated by M@C₆₀, is more abundant than the small quantities of M@C₈₂ species previously available in only milligram quantities via tedious chromatographic separation. Therefore, water-soluble M@C₆₀ species open new opportunities for medical applications of metallofullerenes, since gram quantities of these materials are now readily available.

The objective of the present study was to perform a detailed physicochemical characterization of Gd@C₆₀[C(COOH)₂]₁₀ and Gd@C₆₀(OH)_x in aqueous solution with regard to their efficacy as MRI contrast agents using the same experimental techniques that are usually applied to traditional Gd³⁺-based contrast agents. Based on these systematic measurements, we wished to gain more insight into the paramagnetic relaxation mechanism induced by water-soluble gadofullerenes. Therefore, proton relaxivities have been determined as a function of the magnetic field (NMRD profiles) at different temperatures and at varying pH. Variable-temperature, multiple-field ¹⁷O relaxation rates, and chemical shifts have also been measured for the gadofullerenes in aqueous solutions. Finally, we have made attempts to detect the EPR signals of the gadofullerene derivatives.

Experimental Section

Materials. Gd@C₆₀[C(COOH)₂]₁₀ was produced and characterized as described previously.⁷ A modified polyhydroxylation procedure was conducted on a Gd@C₆₀-containing soot material (synthesis and composition in ref 7) to produce Gd@C₆₀(OH)_x. CH₂Cl₂ (HPLC grade), KO₂ (5–10 mm chunk), and 18-crown-6 (99%) were used as received from Aldrich Chemicals. In a typical synthesis of Gd@C₆₀(OH)_x, a suspension of Gd@C₆₀ (100 mg, ca. 0.1 mmol) and KO₂ (130 mg, 1.8 mmol) was prepared in CH₂Cl₂ (20 mL) with vigorous stirring. To this mixture 18-crown-6 (110 mg, 0.42 mmol; dissolved in 1 mL of CH₂Cl₂) was added dropwise with stirring. Forceful bubbling immediately ensued (evolution of O_{2(g)}), and a brown flocculent precipitate developed, distinct in appearance from both the dark-brown Gd@C₆₀ and yellow KO₂ powders. The mixture was stirred for 1 h and then allowed to settle. The light-brown supernatant was decanted away and the remaining solid was rinsed with CH₂Cl₂ (50 mL), dried in vacuo, and dissolved in distilled water (10 mL) to produce a very dark brown concentrated solution. This solution was passed through a 0.45 μm PTFE filter and concentrated to 1 mL. To this solution was added methanol (10 mL), which induced precipitation of solid Gd@C₆₀(OH)_x. The solid (isolated by centrifugation) was redissolved in water (1 mL), reprecipitated by methanol addition (10 mL), rinsed with methanol (50 mL), and dried under reduced pressure to give 86 mg of dark brown solid. FTIR (KBr matrix, cm⁻¹): 3400 (O–H, v br), 1589, 1372, 1067.

For both Gd@C₆₀[C(COOH)₂]₁₀ and Gd@C₆₀(OH)_x, a single batch was used throughout the NMRD, ¹⁷O NMR, and EPR studies. Recently, we have performed relaxivity measurements on a new batch of gadofullerenol, and under similar conditions, we obtained relaxivities that were identical to previously measured values.

Sample Preparation. The Gd-content of the samples was measured by ICP. In preparation for the ICP measurements, the gadofullerenes were treated with cc. HNO₃ and then carefully heated until a solid residue was obtained. This solid residue was dissolved in 2% HNO₃ and analyzed by ICP: 0.64% (m/m) Gd for Gd@C₆₀(OH)_x and 2.8% (m/m) for Gd@C₆₀[C(COOH)₂]₁₀.

The Gd concentration and the pH of the gadofullerene solutions used were as follows. Gd@C₆₀[C(COOH)₂]₁₀: 3.0 mM, pH = 9.3 (NMRD); 0.45–0.71 mM (pH-dependent relaxivity measurements); 3.6 mM, pH = 3.6 (¹⁷O NMR; EPR). Gd@C₆₀(OH)_x: 0.50 mM, pH = 9.8 (NMRD); 0.074–0.097 mM (pH-dependent relaxivity measurements); 2.7 mM, pH = 3.6 (¹⁷O NMR; EPR). For the pH-dependent relaxometric studies, HClO₄ or NaOH solutions were used to adjust the pH. To increase sensitivity in the ¹⁷O NMR measurements, ¹⁷O enriched water (Isotec, ¹⁷O: 11.4%) was added to the samples to yield a final enrichment of 5%.

All solutions were checked for the presence of free (nonencapsulated) Gd³⁺. Given the deep brown color of the solutions, the usual xylenol orange test is not reliable. Therefore, the proton relaxivities at 60 MHz of the gadofullerene solutions were measured before and after addition of TTHA⁶⁻ (in a 1:1 TTHA⁶⁻: Gd@C₆₀[C(COOH)₂]₁₀ or Gd@C₆₀(OH)_x

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molar ratio with H_6TTHA = triethylenetetramine- N,N,N',N'',N''',N'''' -hexacetic acid). This ligand forms a highly stable complex with Gd^{3+} which contains no inner sphere water molecule.¹² Since $GdTTHA$ would have a much lower relaxivity (from only an outer sphere relaxation mechanism) than any free Gd^{3+} ion, any relaxivity decrease observed upon addition of $TTHA^{6-}$ would signal the presence of free Gd^{3+} ion. In all the gadofullerene samples, the relaxivities with and without $TTHA^{6-}$ were identical, proving the absence of free Gd^{3+} ion.

^{17}O NMR Measurements. Variable-temperature ^{17}O NMR measurements were performed using Bruker spectrometers: AM-400 (9.4 T, 54.2 MHz), Avance-200 (4.7 T, 27.1 MHz), and a 1.41 T, 8.14 MHz electromagnet interfaced with an Avance-200 console. The temperature was measured by a substitution technique.¹³ The samples were sealed in glass spheres and fitted into 10-mm NMR tubes to eliminate susceptibility corrections to the chemical shifts.¹⁴ Longitudinal relaxation rates, $1/T_1$, were obtained by the inversion recovery method, and transverse relaxation rates, $1/T_2$, were measured by the Carr–Purcell–Meiboom–Gill spin–echo technique. As an external reference, acidified water (pH 3.6) was used.

Proton Relaxivity Measurements. The $1/T_1$ NMRD profiles were obtained on a Stellar Spinmaster FFC fast-field-cycling NMR relaxometer covering a continuum of magnetic fields from 5×10^{-4} to 0.47 T (corresponding to a proton Larmor frequency range 0.022–20 MHz). Proton relaxivities at 200 and 400 MHz were measured on Bruker NMR spectrometers; between 20 and 60 MHz they were obtained on a Bruker electromagnet interfaced with an Avance-200 console.

EPR Measurements. The X-band EPR spectra were recorded on a Bruker ESP 300 spectrometer (9.425 GHz, 0.34 T). The spectra at 225 GHz (8.1 T) were recorded on a home-built spectrometer (Department of Experimental Physics, Technical University of Budapest, Hungary).¹⁵ For the home-built spectrometer, a frequency-stabilized Gunn diode oscillator was used at a 75 GHz base frequency, followed by a frequency tripler for the 225 GHz measurements. A PTFE sample holder contained the aqueous sample that was placed in an oversized waveguide, so that no resonant cavity was needed. A Bruker Eleksys E-500 system was used for Q-band (34.0 GHz) measurements.

Results and Discussion

^{17}O NMR Measurements. To explain the high proton relaxivities measured in aqueous solutions for the gadofullerene derivatives, the large number of water molecules on or around the fullerene surface has been generally evoked as being important.^{6,7} In such a relaxation process, the gadofullerene might simultaneously relax the protons of many hydrogen-bonded water molecules on the ca. 200 Å² paramagnetic metallofullerene surface. The efficient proton exchange of these water molecules to the bulk could then transfer the paramagnetic effect of the gadofullerene to bulk water. To investigate if the paramagnetic effect of the gadofullerene derivatives is perceptible to the water oxygen atom, we have measured longitudinal and transverse ^{17}O relaxation rates and chemical shifts at variable temperature and multiple magnetic fields (1.4, 4.7, and 9.4 T) in aqueous solutions for $Gd@C_{60}(OH)_x$ and $Gd@C_{60}[C(COOH)_2]_{10}$. At all temperatures and fields, the chemical shift of the bulk water was identical, within the experimental error, in the gadofullerene solutions and in the acidified water used as a diamagnetic reference. However, the longitudinal relaxation rates measured for both $Gd@C_{60}(OH)_x$ and $Gd@C_{60}[C(COOH)_2]_{10}$ were slightly higher (maximum 15%) than those

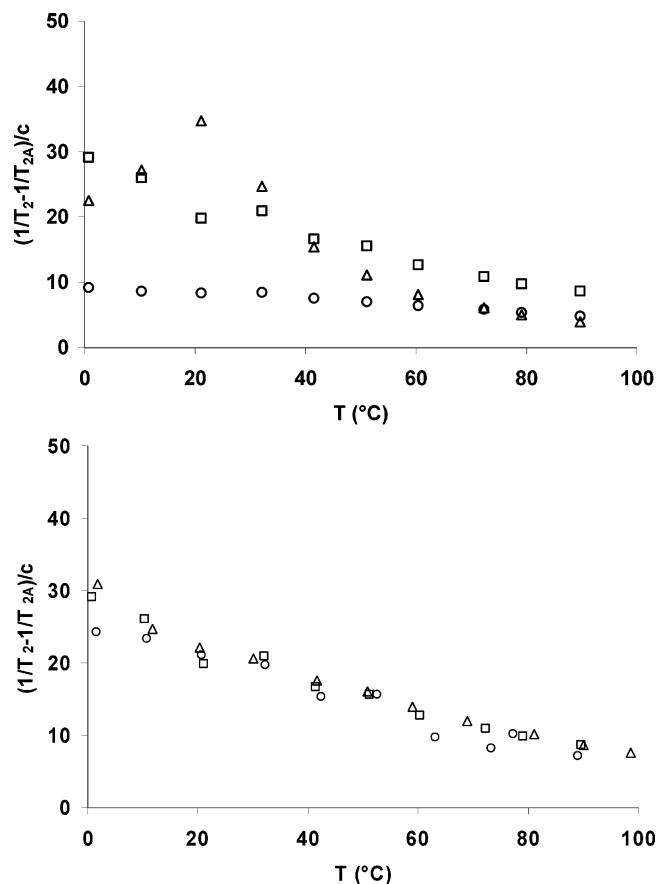


Figure 1. (Top) Enhancement of the transverse ^{17}O relaxation rates (s^{-1}) referred to 1 mM concentration of $Gd@C_{60}[C(COOH)_2]_{10}$ (triangle), $Gd@C_{60}(OH)_x$ (square), and $C_{60}(OH)_x$ (circle). $B = 9.4$ T. (Bottom) Enhancement of the transverse ^{17}O relaxation rates (s^{-1}) referred to 1 mM concentration of $Gd@C_{60}(OH)_x$ at $B = 9.4$ T (square), 4.7 T (triangle), and 1.41 T (circle).

of pure water, but a much more significant influence of the gadofullerenes was observed on the transverse ^{17}O relaxation rates (Figure 1). The effects of $Gd@C_{60}[C(COOH)_2]_{10}$ and $Gd@C_{60}(OH)_x$ on T_2 (referred to unity concentration of the Gd) are similar as shown in Figure 1. It must be noted, however, that even empty $C_{60}(OH)_x$ itself shows a non-negligible effect on the transverse relaxation rates, as also shown in Figure 1.¹⁶ In conclusion, the ^{17}O NMR measurements suggest an “outer-sphere” effect, as would, indeed, be expected for water molecules not directly bonded to the paramagnetic Gd^{3+} center. However, this effect is 10 times larger than that observed for Gd^{3+} poly(aminocarboxylate) complexes without inner sphere water molecules such as $Gd(TETA)^-$ where $H_4TETA = 1,4,8,11$ -tetraazacyclotetradecane-1,4,8,11-tetraacetic acid.¹⁷

Proton Relaxivity Measurements. NMRD profiles ($B = 5 \times 10^{-4}$ to 9.4 T) have been recorded for aqueous solutions of $Gd@C_{60}[C(COOH)_2]_{10}$ and $Gd@C_{60}(OH)_x$ between 278 and 335 K. This is the first report of longitudinal proton relaxivities for gadofullerene derivatives over a large temperature and magnetic field range. The temperature dependence of the proton relaxivities of Gd^{3+} -based contrast agents generally gives very useful information, since the two most important factors that limit

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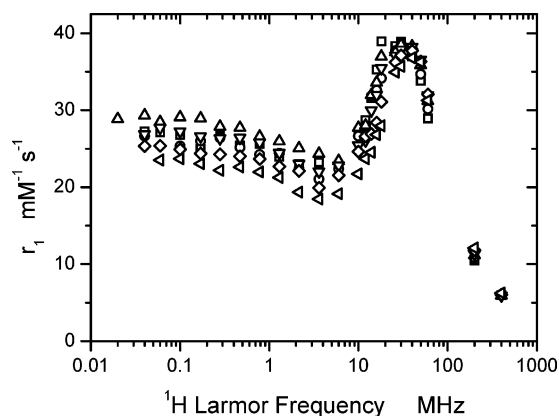


Figure 2. NMRD profiles measured on a Gd@C₆₀(OH)_x ($x \approx 27$) solution ($c_{\text{Gd}} = 0.5 \text{ mM}$; $\text{pH} = 9.8$). Temperatures are 278.0 K (square), 287.5 K (circle), 299.3 K (up pointing triangle), 310.6 K (down pointing triangle), 322.0 K (diamond), and 335.3 K (left pointing triangle).

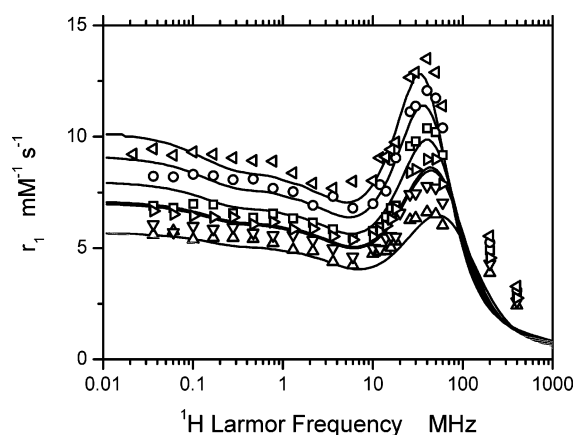


Figure 3. NMRD profiles measured on a Gd@C₆₀[(COOH)₂]₁₀ solution ($c_{\text{Gd}} = 3.6 \text{ mM}$; $\text{pH} = 9.3$). Temperatures are 278.0 K (left pointing triangle), 287.5 K (circle), 299.3 K (square), 310.6 K (right pointing triangle), 322.0 K (down pointing triangle), and 335.3 K (up pointing triangle).

proton relaxivity, the proton exchange rate, and the molecular reorientation have opposite temperature dependence. Therefore, the variation of the proton relaxivities with temperature indicates which of these two factors is limiting. On the other hand, measurement of the proton relaxivity as a function of the magnetic field provides a valuable tool for separating the different interaction mechanisms and dynamic processes influencing the relaxation behavior.

The relaxivities, r_1 , have been calculated from the observed relaxation rates, $1/T_{1,\text{obs}}$, by using eq 1

$$1/T_{1,\text{obs}} = 1/T_{1,d} + r_1 \times [\text{Gd}] \quad (1)$$

where $1/T_{1,d}$ is the diamagnetic contribution and $[\text{Gd}]$ is the concentration of Gd³⁺. The relaxation rate of pure water was taken as the diamagnetic contribution. According to analytical data,⁷ the samples contained mainly Gd@C₆₀[C(COOH)₂]₁₀ or Gd@C₆₀(OH)_x with smaller amounts of malonated or hydroxylated Gd@C₇₀, Gd@C₇₄, and other higher Gd@C_{2n} species as well as C₇₄ (and lesser amounts of C₆₀ and C₇₀). The relaxivity data obtained for Gd@C₆₀(OH)_x and Gd@C₆₀[C(COOH)₂]₁₀ are presented in Figures 2 and 3, respectively.

The relaxivities measured for Gd@C₆₀(OH)_x are particularly large being nearly 10 times greater than those of Gd³⁺-based commercial contrast agents at 20–50 MHz. For both Gd@C₆₀-

(OH)_x and Gd@C₆₀[C(COOH)₂]₁₀ at all temperatures, the NMRD profiles show high field maxima centered at around 40 MHz. Such peaks are usually observed for Gd³⁺ chelates with slow molecular motion.¹⁸ Surprisingly, the high-field (>10 MHz) relaxivities of the Gd@C₆₀(OH)_x are independent of the temperature over the large temperature range studied (278–335 K), whereas they are somewhat temperature dependent at lower frequencies. Similarly, a temperature-independent proton relaxivity was also reported for Gd@C₈₂(OH)_x at 200 MHz between 288 and 328 K.⁶ In contrast, the relaxivities for Gd@C₆₀[C(COOH)₂]₁₀ decrease with increasing temperature at all fields, clearly indicating that slow proton exchange is not relaxivity limiting in this case.

EPR Measurements. In the initial X-band and 225 GHz experiments at room temperature, we did not observe any EPR signal that could be attributed to the gadofullerene derivatives; only a sharp but very weak signal was detected that we assigned to an impurity. In general, this means either very fast electron spin relaxation or the absence of any allowed transitions in the combination of spectral window and observation frequency corresponding to the instrument (X-band: 0–0.6 T, 9.5 GHz; 225 GHz: 7.6–8.6 T). The latter can happen for integer spins with a large zero-field splitting. At Q-band, a very broad spectrum became apparent for the malonate compound at high Gd³⁺ (38 mM) and phosphate buffer (100 mM) concentration. This spectrum can be approximately decomposed into a sum of three Lorentzian derivatives with different widths and integrals. The most obvious feature was the very sharp signal mentioned above (peak-to-peak width ΔH_{pp} less than the modulation amplitude of 8 G), but accounting for less than 0.01% of the integrated intensity. Most of the intensity was found in the other two bands ($\Delta H_{\text{pp}} = 1040 \text{ G}$, 97%; $\Delta H_{\text{pp}} = 160 \text{ G}$, 3%). These two very broad lines provide a good explanation of the apparent lack of signal in more dilute samples.

One possible model for the electronic structure for Gd@C₆₀ involves the transfer of three electrons (5d¹, 6s²) from the gadolinium atom to the cage.^{1,19,20} The resulting molecule carries eight unpaired electrons, one on the cage ($S_{\text{A}} = 1/2$; two transferred electrons paired up) and seven on the metal ($S_{\text{B}} = 7/2$), so that the electron spins can be coupled to produce an effective spin $S' = 3$ or 4. Experimental results (electron energy loss spectroscopy) support this model.²¹ Bertini and co-workers proposed a theoretical description of the electron spin relaxation of coupled systems, from the point of view of EPR or NMR of a nearby nucleus.²² Their description suggests that different effective electron spin relaxation rates might be operational for EPR or for NMR. The relevant equations were originally derived for a total effective spin $1 < S' < 2$ with electron spin relaxation as the only modulation process for the electron–nucleus dipolar interaction. We followed the same procedure for $S' = 7/2 + 1/2 = 4$ and $7/2 - 1/2 = 3$ (see Supporting Information). It is

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straightforward to add other independent modulation processes such as molecular rotation and chemical exchange. Outer sphere relaxation using the free diffusion model of Freed can be treated as well. By using this approach, we attempted to analyze the temperature-dependent proton relaxivities measured for Gd@C₆₀-[C(COOH)₂]₁₀.

Unfortunately, whether the spin-nucleus distance was adjusted or not in the fit of the NMRD profiles, we could not produce a good agreement with the experimental data. In particular, the shallow gap below 10 MHz was never properly reproduced, and its depth was systematically overestimated (a fit example is given in the Supporting Information). Furthermore, the ZFS magnitude parameter Δ_3 and correlation time τ_v for the antiferromagnetic state always indicated a fairly slow electron spin relaxation, similar to what happened when the system was treated as a single $S = 7/2$ spin (typical values obtained in the fit are $\tau_v \approx 10\text{--}20$ ps, $\Delta_3^2 \approx 0.1\text{--}0.2 \times 10^{20} \text{ s}^{-2}$, $\Delta_4^2 \approx (0.2\text{--}1.5) \times 10^{20} \text{ s}^{-2}$). Such parameters should lead to a relatively narrow EPR band, at least for the antiferromagnetic state. The sharp peak in our spectra probably belongs to a paramagnetic impurity among the empty fullerene molecules (similar to the fullerenol radical anion reported by Husebo et al.).¹⁶ However, the other components cannot be easily analyzed. The exchange coupling constant is expected to be very small compared to the Boltzmann energy, so the populations of the ferromagnetic and antiferromagnetic states should be roughly equal. Since the intensities of the two major components do not agree with this assumption, such a simple band attribution seems impossible. Thus we can state this model on its own is not yet able to explain the electron spin relaxation of the gadofullerenes.²³

Fitting the NMRD Profiles. The diameter of a derivatized gadofullerene molecule is relatively large (>10 Å from molecular modeling of Gd@C₆₀[C(COOH)₂]₁₀).⁷ Moreover, it is known that various fullerene derivatives, fullerenols in particular, form aggregates in solution.²⁴ Consequently, the correlation time for rotation rate of this gadofullerene could be in the nanosecond regime. Therefore, we used the program developed by the Florence group²⁵ to perform a simultaneous fit of the six relaxivity profiles measured at different temperatures for Gd@C₆₀[C(COOH)₂]₁₀. We assumed that 20 proton binding sites are available on each molecule (two sites per malonate group) and that the Gd³⁺–H distance for protons bound to a COO[−] group is 6 Å. The closest distance of approach for water protons (for an outer sphere relaxation process) was also assumed to be 6 Å. Equal values for the two distances (Gd³⁺–H bound to carboxylate and Gd³⁺–outer sphere H₂O proton) were used by presuming that bulk H₂O molecules could penetrate to the C₆₀ core in the free space between the malonate groups on the fullerene surface. This analysis of the NMRD profiles yielded the following parameters for Gd@C₆₀[C(COOH)₂]₁₀: $\tau_R^{298} = 2.6$ ns ($E_R = 16$ kJ mol^{−1}), $k_{ex}^{298} = 1.4 \times 10^7 \text{ s}^{-1}$ ($\Delta H^\ddagger = 7.6$ kJ mol^{−1}), $\tau_v^{298} = 11$ ps ($E_R = 3$ kJ mol^{−1}), $\tau_{S0} = 6.6 \times 10^{-10}$ s (which corresponds to $\Delta^2 = 1.1 \times 10^{19} \text{ s}^{-2}$ or 0.0053 cm^{-1}) and a static ZFS of $D = 0.017 \text{ cm}^{-1}$.

For Gd@C₆₀(OH)_x, we could not properly analyze the

variable-temperature NMRD data. In principle, proton exchange and molecular rotation have opposite temperature dependence, whereas electron spin relaxation on Gd³⁺ chelates depends only slightly on temperature. Therefore, a temperature invariance of the proton relaxivities can be the result of compensation between the opposite temperature dependences of water exchange and rotation, as it was indeed observed for a poly(ethylene glycol)-based, Gd^{III} containing polymer.²⁶ For Gd@C₆₀(OH)_x, however, by using sensible activation values for proton exchange, molecular rotation, and electronic relaxation, it was not possible to reproduce the temperature independence of the relaxivity at high magnetic field. Hence, only the experimental data are presented in Figure 2.

As expected for Gd@C₆₀[C(COOH)₂]₁₀, the rotational correlation time is on the order of nanoseconds, and this slow rotation is responsible for the high field maxima in the NMRD profiles. Kato et al. gave a smaller rotational correlation time for Gd@C₈₂(OH)_x ($\tau_R = 0.8$ ns), which was estimated from single-frequency proton relaxivity measurements at 200 MHz.⁶ The proton exchange rate obtained in our analysis is $k_{ex}^{298} = 1.4 \times 10^7 \text{ s}^{-1}$, which is 2–3 times greater than the rate of water exchange from the inner sphere water to bulk water for Gd³⁺ chelates used as MRI contrast agents where proton exchange occurs exclusively via the exchange of an entire water molecule. Proton exchange has been extensively studied for different carboxylic acids by measuring line broadening of the solvent ¹⁷O peak in aqueous solutions. In particular, Leyte et al. reported the rate equation for proton exchange on malonate.²⁷ Using this equation and the conditions of our NMRD experiment, the proton residence time for a COOH group in Gd@C₆₀-[C(COOH)₂]₁₀ in aqueous solution is $\tau_M = 5.1 \times 10^{-8}$ s which corresponds to $k_{ex} = 1.9 \times 10^7 \text{ s}^{-1}$, which is in good agreement with the k_{ex} value obtained above from the NMRD profiles.

pH-Dependent Proton Relaxivities. Figure 4 shows the proton relaxivities measured at 60 MHz for Gd@C₆₀(OH)_x and Gd@C₆₀[C(COOH)₂]₁₀ as a function of the pH over a large pH range (pH = 2–12). For both Gd@C₆₀ derivatives, the relaxivities increase considerably with decreasing pH (by a factor of 2.6 for Gd@C₆₀(OH)_x and 3.8 for Gd@C₆₀[C(COOH)₂]₁₀), until pH ≈ 3 where precipitation initiates. This relaxivity change with pH is completely reversible in the pH range where no precipitation is observed. At low pH's, where precipitation occurs, basification of the precipitated samples within 1–2 h results in immediate dissolution of the solid gadofullerenes and complete recovery of the original relaxivity values. However, keeping the precipitated samples at low pH (below 3) for several hours induces irreversible transformation for both the Gd@C₆₀(OH)_x and Gd@C₆₀[C(COOH)₂]₁₀ samples such that complete dissolution with base is no longer possible.

In clinical MRI, there is an increasing demand for pH-responsive contrast agents. The in vivo pH mapping of tissues could be of importance in future tumor diagnosis in that the pH of solid tumors is approximately 0.4 units lower than that for healthy tissue. Recent studies have shown that the extracellular fluid of tumor cells is acidic, as compared to normal cells. The lower extracellular pH can result from changes in acid elimination from the tumor cells or changes in extracellular

(23) Given the interest in the relaxivity of gadofullerene derivatives from both the practical and theoretical point of view, we feel important to describe our attempts to analyze the experimental proton relaxivities even if these theoretical considerations did not lead to a satisfactory fit of the data.

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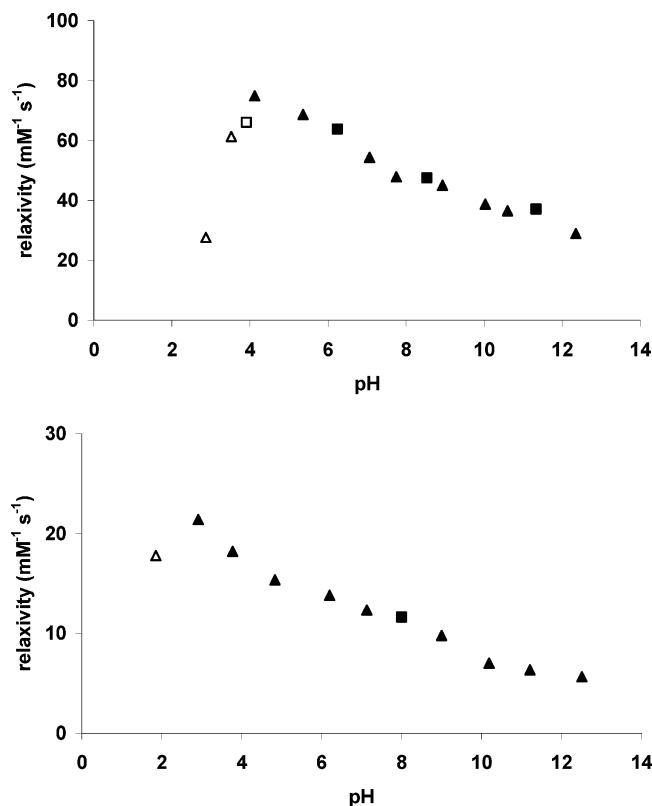


Figure 4. pH dependency of the proton relaxivities for Gd@C₆₀(OH)_x (top) and Gd@C₆₀[C(COOH)₂]₁₀ (bottom) at 60 MHz and 26.1 °C. The triangles represent data obtained by acidifying a basic solution, while the squares show values obtained after adding base to the acidified solution. The empty symbols correspond to solutions with precipitation.

acid clearance and is an intrinsic feature of tumor phenotype.²⁸ Due to its high spatial resolution, magnetic resonance imaging is particularly well suited to studying pH variations over small tissue volumes and, thus, pH-responsive MRI contrast agents such as those of this study are of potential interest for tumor characterization.

Although several contrast agents based on Gd³⁺ poly(aminocarboxylate) complexes have been reported to have pH-dependent relaxivities, so far none of them have proved to be efficient enough for in vivo imaging. A polyionic Gd^{III} complex reported by Mikawa et al. showed relaxivities decreasing from 7.5 to 3 mM⁻¹ s⁻¹ between pH 5 and 7.²⁹ A strong decrease in relaxivity (from 12.8 to 2 mM⁻¹ s⁻¹) between pH 6 and 11 has been found for a positively charged macrocyclic Gd^{III} complex, which was explained with the successive deprotonation of the coordinated water molecules.³⁰ Macromolecular Gd^{III} complexes based on poly(amino acids) such as polylysine or polyornithine have been also proposed for pH assays.³¹ The molecular mobility of these polymers is a function of the pH, resulting in a continuous and linear relaxivity change in a large pH range (for [Gd(DO3ASQ)]₃₀-Orn₁₁₄ *r*₁ increases from 24 to 32 mM⁻¹ s⁻¹ between pH 3–9). A pH-dependent ternary complex formation

between hydrogencarbonate and the Gd^{III} complex of a heptadentate macrocyclic ligand was responsible for its relaxivity decrease from 7.5 to 1.9 mM⁻¹ s⁻¹ between pH 6 and 8.³² Amphiphilic Gd^{III}-DO3A derivatives, containing a tertiary amino group in the side chain, were also reported to have pH-dependent relaxivities (*r*₁ = 7.9 mM⁻¹ s⁻¹ at pH 3–6 and 19.1 mM⁻¹ s⁻¹ at pH 8).³³ This large increase was attributed to the formation of colloidal aggregates, due to the higher lipophilicity of the deprotonated neutral complex at higher pH compared to the protonated and positively charged species at lower pH. More recently, Sherry et al. demonstrated that a methylene nitrophenol arm pending on the Gd^{III} complex may be employed as a pH-responsive unit.³⁴ This pendant arm dissociates from the central lanthanide ion in the pH range 6.5–7.5 causing a relaxivity change from 4.1 mM⁻¹ s⁻¹ at high pH to 7 mM⁻¹ s⁻¹ at low pH.

To explain the origin of the remarkable change in relaxivity of Gd@C₆₀(OH)_x and Gd@C₆₀[C(COOH)₂]₁₀ with pH, two different possibilities must be considered. The pH might influence either the proton exchange rate or the molecular rotation rate, the two main parameters that can limit proton relaxivity. The pH-dependent protonation state of the gadofullerene derivatives can, indeed, affect the rate of the proton exchange. Such effects are well-known for poly(aminocarboxylate) complexes of Gd³⁺.¹⁰ In our case, the fullerene cage is derivatized by functional groups that behave as weak acids. For Gd@C₆₀[C(COOH)₂]₁₀, the temperature dependence of the NMRD curves shows, however, that the relaxivities are not solely limited by proton exchange, since the relaxivity decreases as temperature is increased (Figure 3). This consequently indicates that the observed pH dependence of the relaxivities cannot be explained by a variation of the proton exchange rate.

The rotational motion of the gadofullerenes can also be influenced by pH. Cluster formation or aggregation have been described for solutions of various derivatized fullerenes and for fullerenols in particular.²⁴ The self-assembling aggregation of water-soluble fullerene derivatives may be due to contributions from several aggregation mechanisms including hydrophobic fullerene–fullerene interactions and/or intermolecular hydrogen bonding, with the degree of aggregation varying with pH. Thus, pH can affect the size of the aggregates which will influence the rotational correlation time and consequently the proton relaxivity of the aggregated particles. To learn more about the aggregation properties of Gd@C₆₀(OH)_x and Gd@C₆₀[C(COOH)₂]₁₀ in aqueous solution, we recently performed a comprehensive dynamic and static light scattering (DLS and SLS) study as a function of temperature, concentration and pH.³⁵ The results of this study showed both gadofullerene samples to be highly pH sensitive between pH = 9 and 4, with average hydrodynamic diameters for the aggregates ranging between 70 and 700 nm for Gd@C₆₀[C(COOH)₂]₁₀ and 50 and 1200 nm for Gd@C₆₀(OH)_x. The polydispersity index (PDI) for both

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samples was found to be between 0.4 and 0.5. The variable-temperature DLS studies (288–328 K) showed practically no temperature dependence on average particle size; however, the intensity of the light scattering increased with increasing temperature which was attributed to an increase in the concentration of the aggregates without affecting the particle size. Consequently, the pH dependence of the proton relaxivities for both $\text{Gd@C}_{60}(\text{OH})_x$ and $\text{Gd@C}_{60}[\text{C}(\text{COOH})_2]_{10}$ are a direct result of the pH-dependent aggregation state of the molecules, with the largest particles at the lowest pH's giving rise to the highest relaxivities which are exclusively outer sphere in origin.

Conclusions

Proton relaxivities as a function of temperature and magnetic field have been measured for the water-soluble gadofullerene derivatives, $\text{Gd@C}_{60}(\text{OH})_x$ and $\text{Gd@C}_{60}[\text{C}(\text{COOH})_2]_{10}$. To our knowledge, these are the first full-field nuclear magnetic relaxation dispersion (NMRD) profiles to be reported for endohedral gadofullerenes. For both derivatives, the proton relaxivities show high-field peaks, typical of slowly rotating, Gd^{3+} -containing species, with maximum relaxivities of $10.4 \text{ mM}^{-1} \text{ s}^{-1}$ for $\text{Gd@C}_{60}[\text{C}(\text{COOH})_2]_{10}$ and $38.5 \text{ mM}^{-1} \text{ s}^{-1}$ for $\text{Gd@C}_{60}(\text{OH})_x$ around 30–60 MHz at 299 K. The relaxation mechanism generating these high relaxivities is entirely outer-sphere in nature, and as such, they represent the largest outer sphere proton relaxivities ever recorded for a Gd^{3+} -based MRI contrast agent. An analysis of the NMRD profiles for $\text{Gd@C}_{60}[\text{C}(\text{COOH})_2]_{10}$ resulted in a rotational correlation time of $\tau_R^{298} = 2.6 \text{ ns}$ and a proton exchange rate of $k_{\text{ex}}^{298} = 1.4 \times 10^7 \text{ s}^{-1}$; this latter value is consistent with the proton exchange rate previously determined for malonic acid. The proton relaxivities display a remarkable pH dependency, increasing dramatically

with decreasing pH. For both $\text{Gd@C}_{60}(\text{OH})_x$ and $\text{Gd@C}_{60}[\text{C}(\text{COOH})_2]_{10}$, this trend has been related to aggregation phenomena, as established by DLS/SLS measurements. The strong pH dependency of the proton relaxivities makes these gadofullerene derivatives excellent candidates for pH-responsive MRI contrast agent applications. Gadofullerenes could be especially valuable in this application because water-soluble fullerenes (such as the neuroprotective drug, C_{33}^{36}) readily cross cell membranes, suggesting their application as intracellular MRI agents as well as pH-responsive ones.

Acknowledgment. This research was supported in part by the Swiss National Science Foundation and the Office for Education and Science (OFES) and was carried out in the frame of the EC COST Action D18. R.D.B. and L.J.W. also thank the U.S. National Institutes of Health (Grant NIH 1 R01 EB00703-01) and the Robert A. Welch Foundation (Grant C-0627/L.J.W.) for support of this work.

Supporting Information Available: Proton relaxivities as a function of magnetic field, temperature, and pH; transverse and longitudinal ^{17}O relaxation rates and chemical shifts; typical parameters and experimental and simulated NMRD profiles obtained from a fit of $\text{Gd@C}_{60}[\text{C}(\text{COOH})_2]_{10}$ NMRD profiles assuming coupled electron spins on Gd^{3+} ($S = 7/2$) and the fullerene cage ($S = 1/2$); theory of nuclear and electron spin relaxation for an $S = 7/2 + S = 1/2$ system. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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