The Role of Water Exchange in Attaining Maximum Relaxivities for Dendrimeric MRI Contrast Agents**

Abstract: Macrocyclic Gd^{III} complexes attached to dendrimers represent a new class of potential MRI contrast agents. They have an extended lifetime in the blood pool, which is indispensable for their application in magnetic resonance angiography, and high relaxivities, which reduce the dose required to produce quality images. We performed a variable-temperature and -pressure ^{17}O NMR study in aqueous solution and at 14.1, 9.4, and 1.4 T on the water exchange and rotational dynamics of three macrocyclic Gd^{III} complexes based on polyamidoamine dendrimers, as well as on the Gd^{III} complex of the monomer unit with the linker group. The water exchange rates k_{ex}^{298} for generation 5 [G5(N{CS}N-bz-Gd-{DO3A}{H_2O})$_{52}$], generation 4 [G4(N-{CS}N-bz-Gd{DO3A}{H_2O})$_{30}$], generation 3 [G3(N{CS}N-bz-Gd{DO3A}-{H_2O})$_{23}$], and the monomer [Gd(DO3A-bz-NO_2)(H_2O)] complexes are 1.5 ± 0.1, 1.3 ± 0.1, 1.0 ± 0.1, and $1.6\pm0.1\times10^6\ s^{-1}$, respectively, and the activation volumes $\Delta V^{\neq}$ of water exchange on the latter two compounds are $+3.1\pm0.2$ and $+7.7\pm0.5\ cm^3\ mol^{-1}$, indicating dissociatively activated exchange reactions ({CS}N-bz-{DO3A} = 1-(4-isothiocyanatobenzyl)amido-4,7,10-tri(acetic acid)tetraazacyclododecane). The rotational correlation times for the dendrimers are 4 to 8 times longer than for monomeric or dimeric Gd^{III} poly(amino carboxylates). As a consequence of the slow rotation, the proton relaxivities of these dendrimer complexes are considerably higher than those of smaller complexes. However, the low water exchange rates prevent the dendrimer proton relaxivities from attaining the values expected from the increase in the rotational correlation times. Modifications of the chelating ligand may result in a faster water exchange and thus allow the full benefit of slow rotation to be achieved.

Keywords
contrast agents · dendrimers · gadolinium complexes · ligand exchange · magnetic resonance imaging

Introduction

Since magnetic resonance imaging (MRI) was introduced as one of the most powerful tools in medical diagnostics, there has been a continuous interest in the design, synthesis, and characterization of Gd^{III} complexes as possible contrast agents in MRI.[2] Most recently a new and very promising application of this technique, magnetic resonance angiography (MRA), has presented a substantial challenge for the development of new types of Gd-based contrast agents.[3] Contrast agents are required that, unlike the commercially available low molecular weight Gd chelates, remain in the blood vessels for an extended period of time. As a result, it is also desireable that they have high relaxivities, so that the required doses of the contrast agent can be lowered and safety thus increased. Macromolecular contrast agents are the best candidates for this purpose as they are expected to fulfil both requirements.[4]

The efficiency of an MRI contrast agent—its ability to accelerate proton relaxation over the pure water or serum background—is measured by its relaxivity. Overall relaxivity is determined by two terms: 1) short-range dipolar interactions between the unpaired electron spins and the proton nuclei of water molecules bound in the first coordination sphere of the paramagnetic metal, mediated to the bulk by chemical exchange (inner-sphere relaxation), and 2) long-range dipolar interactions between the paramagnetic metal ion and the bulk water in the vicinity of the complex (outer-sphere relaxation). For monomeric Gd complexes currently used as contrast agents, which contain one inner-sphere water molecule, these two mechanisms contribute to the overall relaxivity to approximately the same extent.[2, 5] The inner-sphere relaxivity is governed by the rotational correlation time of the complex (τ_R), the residence time of a water proton in the inner coordination sphere τ_m (or its inverse, the exchange rate $k_{ex}=1/\tau_m$), and the longitudinal and transverse electronic relaxation times ($T_{1,2e}$). The proton residence time, at least at neutral pH, equals the residence time of the oxygen nucleus, since proton exchange at neutral pH takes place through the exchange of whole water molecules, as was confirmed recently (e.g., for Gd(DTPA-BMA)(H_2O),[6, 7] where DTPA-BMA = 1,7-bis[(*N*-methylcarbamoyl)methyl]-1,4,7-triazaheptane-1,4,7-triacetate). These parameters that are of high importance in proton relaxivity have already been determined for several monomeric and dimeric Gd chelates; this helps in the understanding of the function of these (potential) contrast agents as well as in the design of new and better ones. In these studies ^{17}O NMR spectroscopy is a highly powerful

Institut de Chimie Minérale et Analytique
Université de Lausanne, BCH, CH-1015 Lausanne (Switzerland)
Fax: Int code +(21)692-3875

[**] High-Pressure NMR Kinetics, Part 72; part 71, see ref. [1].

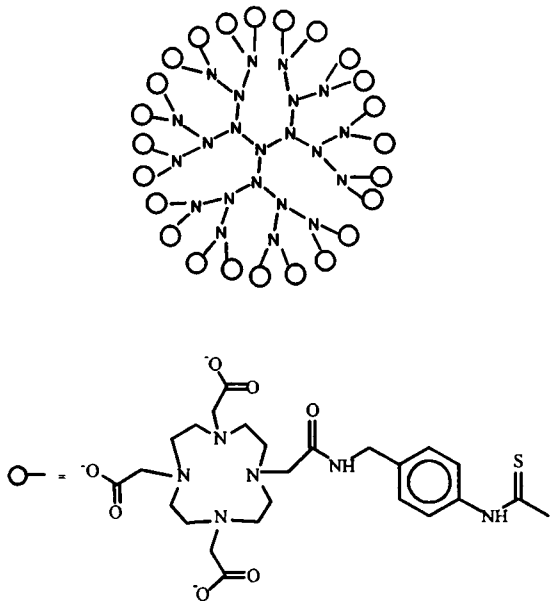
  0947-6539/96/0212-1607 $ 15.00+.25/0

method, as the oxygen atom of the coordinated water molecule is directly bound to the paramagnetic ion and is thus a more sensitive antenna than the protons.[8] The other advantage of this technique is that the outer-sphere contribution to ^{17}O relaxation is negligible.[9]

Macrocyclic Gd complexes attached to dendrimers are promising candidates to meet the needs of blood pool agents.[10-12] Dendrimers are highly ordered three-dimensional polymers[13-15] that have several advantages over linear polymers: their polydispersity is very low, their size, shape, and loading with Gd chelates are easily controlled. The first results obtained with a dendrimer-based Gd(DTPA)-type contrast agent showed very good imaging properties[16] (DTPA = diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetate). The introduction of a macrocyclic tetraazapolyacetate ligand instead of DTPA further improved the kinetic inertness and thermodynamic stability of the complex.

These dendritic Gd complexes possess several positive features beside prolonged intravascular retention. First of all the rotational correlation time of the macromolecule as a whole is long. This approach towards higher relaxivities (i.e., increasing the rotational correlation time by increasing the molecular weight) has been used previously with the introduction of dimeric contrast agents,[17, 18] or with the linkage of Gd^{III} chelates to large biomolecules, such as dextrans,[19] polylysine, or serum albumin.[20] In other cases the Gd^{III} complex was incorporated into the polymer chain.[21] However, for linear polymers the proton relaxivity was generally not increased as much as expected. The relaxivity gain that would arise from the long rotation of the whole molecule is partially lost owing to internal rotational motions of the flexible chains, which can be much faster than rotation of the entire molecule for high molecular weight compounds.[5] In contrast to linear polymers, dendrimers have a rather rigid structure, and the overall tumbling of the molecule contributes to the rotational correlation time.[22] Furthermore, in the molecules investigated in this study the macrocylic ligand that binds the Gd^{3+} is attached to the dendrimer through a short and rigid linker to avoid a rapid rotational motion of the chelate itself, so that the slower rotional motion of the entire molecule is the sole contributor to τ_R. This strategy enables the highest possible relaxivities to be attained. The macrocycle contains an amide group in the linker, which also contributes to the kinetic and thermodynamic stability of the complex, as it is coordinated to the lanthanide ion. Finally, the molecule as a whole is not charged; this ensures a lower osmolality and thus less painful injection.

In order to help synthetic chemists to develop new types of MRI contrast agents, it would be helpful to know how the parameters describing water exchange, which might limit the relaxivity of a Gd^{III} complex, are affected when the latter is attached to a large molecule (e.g., to a dendrimer). Furthermore, this information would increase our general understanding of how MRI contrast agents work. We therefore performed a variable-temperature and variable-pressure ^{17}O NMR study in aqueous solution at three different magnetic fields (1.41, 9.4, and 14.1 T) on the complex -{CS}N-bz-Gd{DO3A}{H_2O} attached to three different generations of polyamidoamine (PAMAM) type starburst dendrimers:[13] generation 5 [G5-(N{CS}N-bz-Gd{DO3A}{H_2O})$_{52}$] (G_5Gd_{52}), generation 4 [G4(N{CS}N-bz-Gd{DO3A}{H_2O})$_{30}$] (G_4Gd_{30}), and generation 3 [G3(N{CS}N-bz-Gd {DO3A}{H_2O})$_{23}$] (G_3Gd_{23}; Scheme 1) ({CS}N-bz-{DO3A} = 1-(4-isothiocyanatobenzyl)-methylcarbamoyl-4,7,10-tri(acetic acid)tetraazacyclododecane) (Scheme 1). This is the first time that parameters characterizing the water exchange and the rotational dynamics as seen by ^{17}O



Scheme 1. Structure of the third-generation dendrimeric ligand, [G3(N{CS}N-bz-{DO3A})$_{23}$].

NMR are reported for a *macromolecular* Gd-based potential contrast agent. As a comparison, the complex [Gd(DO3A-bz-NO_2)(H_2O)], which represents the monomeric chelate unit of the dendritic complexes, was also investigated (DO3A-bz-NO_2 = 1-(4-nitrobenzyl)methylcarbamoyl-4,7,10-tri(acetic acid)-tetraazacyclododecane).

Results

^{17}O NMR Spectroscopy: From the measured ^{17}O NMR relaxation rates and angular frequencies of the paramagnetic solutions ($1/T_1$, $1/T_2$, and ω) and of the acidified water reference ($1/T_{1A}$, $1/T_{2A}$, and ω_A) one can calculate the reduced relaxation rates and chemical shift ($1/T_{1r}$, $1/T_{2r}$, and $\Delta\omega_r$), according to Equations (1-3),[23] where $1/T_{1m}$ and $1/T_{2m}$ are the relaxation

$$\frac{1}{T_{1r}} = \frac{1}{P_m}\left[\frac{1}{T_1} - \frac{1}{T_{1A}}\right] = \frac{1}{T_{1m} + \tau_m} + \frac{1}{T_{1os}} \quad (1)$$

$$\frac{1}{T_{2r}} = \frac{1}{P_m}\left[\frac{1}{T_2} - \frac{1}{T_{2A}}\right] = \frac{1}{\tau_m}\frac{T_{2m}^{-2} + \tau_m^{-1}T_{2m}^{-1} + \Delta\omega_m^2}{\left(\tau_m^{-1} + T_{2m}^{-1}\right)^2 + \Delta\omega_m^2} + \frac{1}{T_{2os}} \quad (2)$$

$$\Delta\omega_r = \frac{1}{P_m}(\omega - \omega_A) = \frac{\Delta\omega_m}{\left(1 + \tau_m T_{2m}^{-1}\right)^2 + \tau_m^2\Delta\omega_m^2} + \Delta\omega_{os} \quad (3)$$

rates in the bound water, $\Delta\omega_m$ is the chemical shift difference between bound and bulk water (in the absence of a paramagnetic interaction with the bulk water), P_m is the mole fraction of bound water, and τ_m is the residence time of water molecules in the inner coordination sphere. The total outer-sphere contributions to the reduced relaxation rates and chemical shift are represented by $1/T_{1os}$, $1/T_{2os}$, and $\Delta\omega_{os}$.

It has been shown that the outer-sphere contributions in Equations (1) and (2) can be neglected.[9] The maxima observed in the temperature dependence of $1/T_{2r}$ are characteristic of a changeover from the "fast exchange" limit at high temperatures, where T_{2m} is the dominant term in the denominator of Equa-

0947-6539/96/0212-1608 $ 15.00+.25/0 Chem. Eur. J. 1996, 2, No. 12

tion (2), to the "slow exchange" limit at low temperatures, where τ_m is the dominant term. Since $T_{1m} > T_{2m}$, the maximum in $1/T_{1r}$ is shifted to lower temperatures as can be seen in the results in Figures 1–4.

The changeover between fast and slow exchange limits is also manifested in $\Delta\omega_r$ and the maxima in the plot of $1/T_{2r}$, corresponding to the points of inflection in the plots of $\Delta\omega_r$. At high temperatures, the inner-sphere contribution to $\Delta\omega_r$ is given by the chemical shift of the bound water molecules, which is determined by the hyperfine interaction between the Gd^{3+} electron spin and the ^{17}O nucleus as shown in Equation (4), where g_L is

$$\Delta\omega_m = \frac{g_L \mu_B S(S+1) B}{3k_B T} \frac{A}{\hbar} \quad (4)$$

the isotropic Landé g factor ($g_L = 2.0$ for Gd^{3+}), S is the electron spin ($S = 7/2$ for Gd^{3+}), $A/\hbar$ is the hyperfine or scalar coupling constant, and B is the magnetic field.[24] We assume that the outer-sphere contribution to $\Delta\omega_r$ has a similar temperature dependence as $\Delta\omega_m$ and is given by Equation (5), where C_{os} is an empirical constant.

$$\Delta\omega_{os} = C_{os}\Delta\omega_m \quad (5)$$

The ^{17}O longitudinal relaxation rates in Gd^{3+} solutions are dominated by the dipole–dipole and quadrupolar mechanisms [Eq. (6)].[25] Owing to the relatively long rotational correlation

$$\frac{1}{T_{1m}} = \frac{1}{T_{1dd}} + \frac{1}{T_{1q}} \quad (6)$$

times, τ_R, we used a full treatment that also included nonextreme narrowing conditions. The dipolar longitudinal relaxation is given by Equation (7).[26] For the quadrupolar relaxation we

$$\frac{1}{T_{1dd}} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\hbar^2\gamma_I^2\gamma_s^2}{r^6} S(S+1)\left[\frac{7\tau_C}{1+\omega_s^2\tau_C^2} + \frac{3\tau_C}{1+\omega_I^2\tau_C^2}\right] \quad (7)$$

used the approximation developed by Halle et al. [Eq. (8)],[27] where $\gamma_s = g_L\mu_B/\hbar$ is the electron gyromagnetic ratio ($\gamma_s = 1.76 \times 10^{11}$ rad s^{-1} T^{-1} for $g_L = 2.0$), γ_I is the nuclear gyromag-

$$\frac{1}{T_{1q}} = \frac{3\pi^2}{10}\frac{2I+3}{I^2(2I-1)}\chi^2(1+\eta^2/3)\left[\frac{0.2\tau_R}{1+\omega_I^2\tau_R^2} + \frac{0.8\tau_R}{1+4\omega_I^2\tau_R^2}\right] \quad (8)$$

netic ratio ($\gamma_I = -3.626 \times 10^7$ rad s^{-1} T^{-1} for ^{17}O), r is the effective distance between the electron spin and the ^{17}O nucleus (the metal–oxygen distance in the point-dipole approximation), I is the nuclear spin ($I = 5/2$ for ^{17}O), ω_s is the electron and ω_I the nuclear spin resonance frequency, χ is the quadrupolar coupling constant, and η an asymmetry parameter (we use here the value for acidified water, $\chi(1+\eta^2/3)^{1/2} = 7.58$ MHz).[28] For the effective distance we use the same value as in all the previous ^{17}O NMR studies of Gd^{III} complexes[6, 9, 17, 25] ($r = 0.25$ nm), which is estimated from neutron diffraction measurements of lanthanide(III) aqua ions in solution. With this r value, the dipole–dipole mechanism contributes ca. 70% of $1/T_{1m}$. The correlation time, τ_c, is characteristic for the dipolar longitudinal relaxation, and can be expressed by Equation (9), where τ_R is the rotational correlation time and T_{1e} is longitudinal electronic relaxation time. The two last terms in Equation (9) are small compared to $1/\tau_R$, and can therefore be neglected.[29]

$$1/\tau_c = 1/\tau_R + 1/T_{1e} + 1/\tau_m \quad (9)$$

We assume that the correlation time τ_R has a simple exponential temperature dependence as given in Equation (10), where

$$\tau_R = \tau_R^{298} \exp\left\{\frac{E_R}{R}\left(\frac{1}{T} - \frac{1}{298.15}\right)\right\} \quad (10)$$

τ_R^{298} is the rotational correlation time at 298.15 K and E_R the activation energy of rotation. The binding time (or exchange rate, k_{ex}) of water molecules in the inner sphere is assumed to obey the Eyring equation as depicted in Equation (11), where

$$\frac{1}{\tau_m} = k_{ex} = \frac{k_B T}{h}\exp\left\{\frac{\Delta S^*}{R} - \frac{\Delta H^*}{RT}\right\} = \frac{k_{ex}^{298} T}{298.15}\exp\left\{\frac{\Delta H^*}{R}\left(\frac{1}{298.15} - \frac{1}{T}\right)\right\} \quad (11)$$

ΔS^* and ΔH^* are the entropy and enthalpy of activation for the exchange process, and k_{ex}^{298} is the exchange rate at 298.15 K. The transverse relaxation rate of the bound water $1/T_{2m}$ was assumed to follow an exponential temperature dependence as shown in Equation (12), where E_m is the activation energy of the relaxation of the bound water and $1/T_{2m}^{298}$ the relaxation rate of the bound water at 298.15 K.

$$\frac{1}{T_{2m}} = \frac{1}{T_{2m}^{298}}\exp\left\{\frac{E_m}{R}\left(\frac{1}{298.15} - \frac{1}{T}\right)\right\} \quad (12)$$

A more detailed approach to the ^{17}O transverse relaxation rate of the bound water in Gd^{3+} solutions, which is dominated by the scalar relaxation mechanism, incorporates the parameters for the electronic relaxation.[6, 25, 30] This full treatment, however, requires reliable values for the parameters characterizing the electron spin relaxation, which can only be obtained from EPR measurements.[30] Owing to the lack of EPR data, no satisfactory fit could be obtained for the dendritic complexes when electronic relaxation parameters were adjusted only on the basis of the ^{17}O NMR results. We therefore used the simple exponential model given above for describing the transverse relaxational rates. The validity of this simplified treatment has been verified by refitting previously published ^{17}O NMR data for [Gd(DTPA-BMA)(H_2O)][6] with Equation (12), and the resulting kinetic parameters were unchanged within statistical error.

We performed a simultaneous least-squares fit of the data in Figures 1–4 using Equations (1–12) with the following fitted parameters: k_{ex}^{298} (or ΔS^*), ΔH^*, $A/\hbar$, C_{os}, τ_R^{298}, E_R, $1/T_{2m}^{298,HF}$, $1/T_{2m}^{298,LF}$, and E_m. The resulting curves are shown in Figures 1–4 and the fitted parameters are given in Table 1. Since there was no field dependence of the reduced transverse relaxation rates between 9.4 and 14.1 T, these data were fitted with a single value for the bound water relaxation at 298 K ($1/T_{2m}^{298,HF}$, HF for high field, $B = 14.1$ and 9.4 T), while for low fields ($B = 1.4$ T) the corresponding parameter $1/T_{2m}^{298,LF}$ was used. E_m was fitted to a single value for all three fields. No concentration dependence was found for the reduced transverse and longitudinal relaxation rates and chemical shifts for G_3Gd_{23}, and the $1/T_{2r}$, $1/T_{1r}$, and $\Delta\omega_r$ data obtained in solutions of two different concentrations were thus fitted together.

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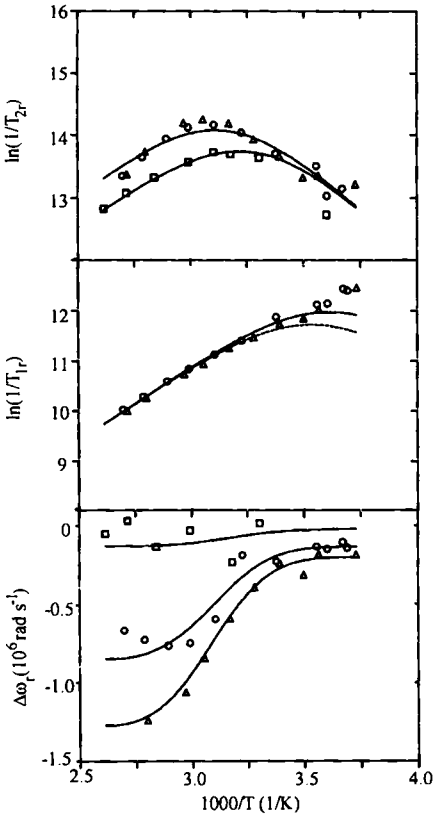

Fig. 1. Temperature dependence of the reduced ^{17}O transverse and longitudinal relaxation rates ($1/T_{1r}$ and $1/T_{2r}$, s^{-1}) and chemical shifts ($\Delta\omega_r$) for aqueous solutions of [G5(N{CS}N-bz-Gd{DO3A}{H_2O})$_{52}$] at 1.41 (□), 9.4 (○), and 14.1 T (△). The lines are functions calculated from a simultaneous nine-parameter least-squares fit (see text). For the $\ln(1/T_{1r})$ values, the dashed curve corresponds to $B = 14.1$ T, and the solid curve to $B = 9.4$ T.

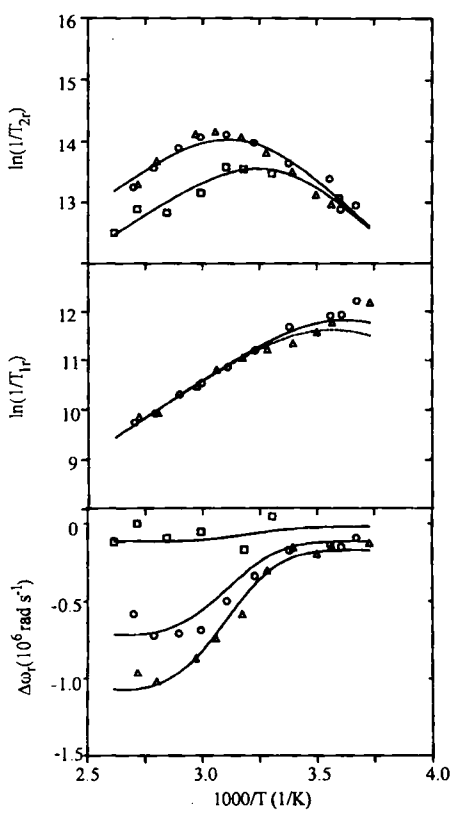

Fig. 2. Same as Fig. 1 but for [G4(N{CS}N-bz-Gd{DO3A}{H_2O})$_{30}$].

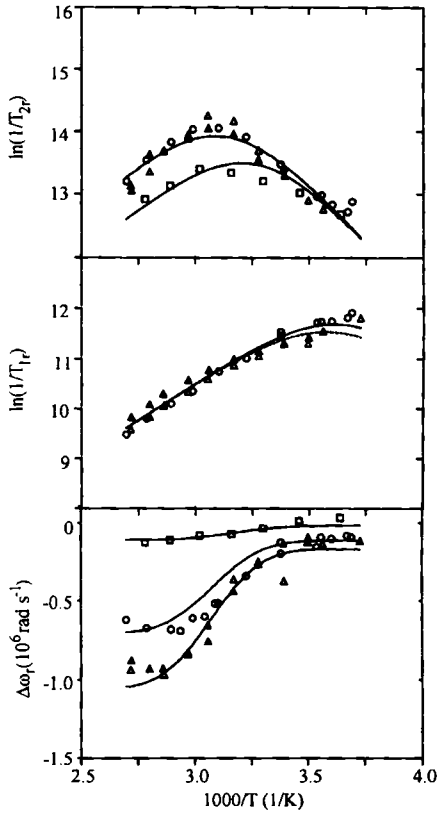

Fig. 3. Same as Fig. 1 but for [G3(N{CS}N-bz-Gd{DO3A}{H_2O})$_{23}$]; at 14.1 T: $c_{Gd} = 0.0996$ M (△), $c_{Gd} = 0.1706$ M (▲).

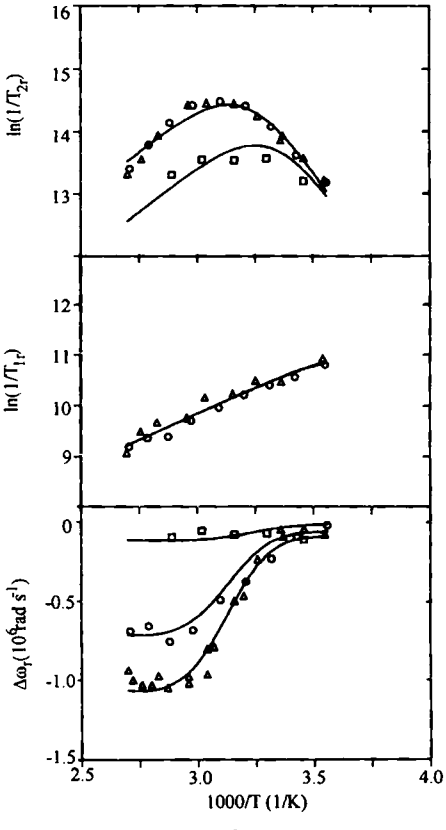

Fig. 4. Same as Fig. 1 but for [Gd(DO3A-bz-NO_2)(H_2O)].

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The pressure dependence of the reduced transverse relaxation rates $1/T_{2r}$ for [G3(N{CS}N-bz-Gd{DO3A}{H_2O})$_{23}$] and [Gd(DO3A-bz-NO_2)(H_2O)] at 278.2 and 288.6 K, respectively, and at 9.4 T is shown in Figure 5. At this magnetic field and the temperatures chosen, the systems are in the slow exchange limit, and $1/T_{2r}$ is close to $1/\tau_m$. The decrease of $1/T_{2r}$ with pressure in Figure 5 is, therefore, due to a slowing of the water exchange process. The pressure dependence of the water exchange rate may be written as shown in Equation (13), where $\Delta V_0^{\neq}$ is the

$$\frac{1}{\tau_m} = k_{ex} = (k_{ex})_0^T \exp\left\{-\frac{\Delta V_0^r}{RT}P + \frac{\Delta \beta^r}{2RT}P^2\right\} \quad (13)$$

activation volume at zero pressure and temperature T, $(k_{ex})_0^T$ is the exchange rate at zero pressure and temperature T, and $\Delta\beta^{\neq}$ is the compressibility coefficient of activation. As in previous studies, the pressure dependence of $\ln(k_{ex})$ has been found to be nearly linear,[6, 9] so we assume that $\Delta\beta^{\neq} = 0$ and that we have an activation volume independent of pressure ($\Delta V^{\neq}$ instead of $\Delta V_0^{\neq}$). The scalar coupling constant was found previously to be independent of pressure,[31] so we assume that it is constant and equal to the value in Table 2. In the fitting procedure we included a possible pressure dependence of the bound water relaxation rate, $1/T_{2m}$, as given in Equation (14). We performed a least-squares fit of the

$$\frac{1}{T_{2m}} = \frac{1}{T_{2m}^0}\exp\frac{-\Delta V_m^r}{RT}P \quad (14)$$

data in Figure 5 using Equations (2), (4), and (12–14) with $(k_{ex})_0^T$ and $\Delta V^{\neq}$ as fitted parameters. Fixing $\Delta V_m^{\neq}$ at values of +5 and −5 cm^3 mol^{-1} did not alter the values in Table 2 within the statistical error; this was expected since, at the temperature chosen for the variable-pressure measurements, k_{ex} contributes more than 90% to $1/T_{2r}$. The fitted functions are shown in Figure 5, and the fitted parameters are given in Table 1.

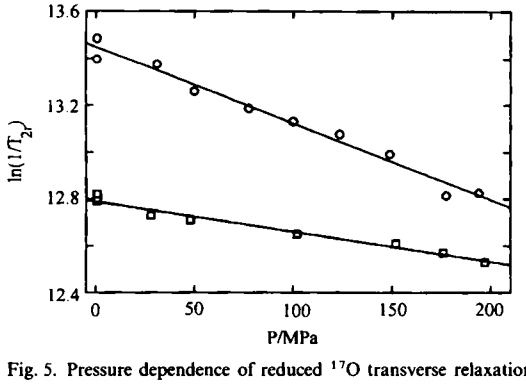

Fig. 5. Pressure dependence of reduced ^{17}O transverse relaxation rates (s^{-1}) for aqueous solutions of [G3(N{CS}N-bz-Gd{DO3A}{H_2O})$_{23}$] at 278.2 K (□) and [Gd(DO3A-bz-NO_2)(H_2O)] at 288.6 K (○) (B = 9.4 T).

Discussion

The solution structure of the first coordination sphere for the lanthanide(III) ions in the dendrimer complexes was investigated by UV/Vis spectrophotometry for europium and by the water ^{17}O shift induced by the contact interaction between the paramagnetic gadolinium ion and the bound water.

Table 1. Kinetic and NMR parameters obtained from ^{17}O relaxation, and chemical shift data as a function of temperature and pressure.

	G_5Gd_{52}	G_4Gd_{30}	G_3Gd_{23}	Gd(DO3A-bz-NO_2)
$k_{ex}^{298} \times 10^{-6}$ (s^{-1})	1.5±0.1	1.3±0.1	1.0±0.1	1.6±0.1
$\Delta H^{\neq}$ (kJ mol^{-1})	24.0±2.5	27.7±2.6	28.8±2.2	40.9±2.5
$\Delta S^{\neq}$ (J mol^{-1})	−43±15	−31±10	−30±10	+11.1±8
$\Delta V^{\neq}$ (cm^3 mol^{-1})			+3.1±0.2	+7.7±0.5
$A/\hbar \times 10^{-6}$ (rad s^{-1})	−4.6±0.5	−3.9±0.4	−3.9±0.3	−3.8±0.2
$\tau_R^{298} \times 10^{12}$ (s)	870±20	700±20	580±10	210±10
E_R (kJ mol^{-1})	24.8±0.2	25.5±0.1	23.6±0.1	17.7±0.1
C_{os}	0.11±0.08	0.11±0.07	0.11±0.05	0.06±0.04
$1/T_{2m}^{298,LF} \times 10^{-6}$ (s)	2.4	1.7	1.7	2.0
$1/T_{2m}^{298,HF} \times 10^{-6}$ (s)	3.3	3.2	2.9	5.1
E_m (kJ mol^{-1})	20.1±2.4	20.6±2	20.9±3	24.4±2.3

Table 2. A comparison of the water exchange rates, activation volumes, rotational correlation times, and proton relaxivities for different Gd^{III} complexes.

	$k_{ex}^{298} \times 10^{-6}$ (s^{-1})	$\Delta V^{\neq}$ (cm^3 mol^{-1})	$\tau_R^{298} \times 10^{12}$ (s)	R_1 (mM^{-1} s^{-1}) (20 MHz; 37 °C)
$[Gd(H_2O)_8]^{3+}$ [25]	830±95	−3.3±0.2	29±2	
$[Gd(DTPA)(H_2O)]^{2-}$ [9]	4.1±0.3	+12.5±0.2	103±10	4.02 [a] [18]
[Gd(DTPA-BMA)(H_2O)] [6]	0.43±0.2	+7.3±0.2	167±5	3.96 [a] [18]
$[Gd(DOTA)(H_2O)]^-$ [9]	4.8±0.4	+10.5±0.2	90±15	3.83 [b] [49]
[Gd(DO3A-bz-NO_2)]	1.6±0.1	+7.7±0.5	211±8	
[BO{Gd(DO3A)(H_2O)}$_2$] [17]	1.0±0.1	+0.5±0.2	250±5	4.61 [56]
[pip{Gd(DO3A)(H_2O)}$_2$] [18]	1.5±0.1		270±9	5.79
[bisoxa{Gd(DO3A)(H_2O)}$_2$] [18]	1.2±0.1	+2.2±0.2	220±7	4.94
G_3Gd_{23}	1.0±0.1	+3.1±0.2	580±10	14.57 [10]
G_4Gd_{30}	1.3±0.1		700±20	
G_5Gd_{52}	1.5±0.1		870±20	18.71 [10]

[a] t = 35 °C. [b] t = 39 °C.

Differences in the coordination of a Eu^{3+} ion are manifested by shifts in the $^7F_0 \rightarrow {}^5D_0$ transition band, and the presence of a coordination equilibrium therefore results in two absorptions that change their relative intensity with temperature. A single $^7F_0 \rightarrow {}^5D_0$ band is observed for G_5Eu_{52}. Its temperature invariance shows that there is no equilibrium between species with a different number of water molecules in an aqueous solution of the Eu complex. By analogy to lanthanide poly(amino carboxylates) previously investigated,[32–35] we assume that, in the only species present in solution, the lanthanide ion is coordinated to nine atoms: four nitrogens of the macrocyclic ring, four oxygen atoms of the carboxylate arms and of the amide moiety, and one water molecule. The coordination of the amide group has already been demonstrated for Ln^{3+} complexes of different amide derivatives of DTPA and DOTA[36–39] (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate). This coordination mode can accommodate one water molecule in the inner sphere, and this number does not change in the temperature range used for the spectrophotometric and ^{17}O NMR studies. The position of the $^7F_0 \rightarrow {}^5D_0$ transition band predicted on the basis of this structure by the empirical equation of Horrocks et al.[40] is similar to what was found experimentally for [G5(N{CS}N-bz-Eu {DO3A}{H_2O})$_{52}$] (λ_{calcd} = 579.78 nm; λ_{exp} = 579.71 ± 0.05 nm). As all three dendrimer and the monomer ligands have exactly the same macrocyclic unit to coordinate the lanthanide ion, this finding was extrapolated for the Eu^{III} and the Gd^{III} complexes of the generation 4 and 3 dendrimers and of the DO3A-bz-NO_2 monomer. The value of the scalar coupling constant ($A/\hbar$) obtained by ^{17}O NMR (Table 1) is also in the usual range for Gd^{III} complexes with one inner-sphere water molecule (−3.4 to -5.3×10^6 rad s^{-1}).[6, 9, 17, 18, 25] Therefore we can be confident that there is one inner-sphere water molecule in these complexes.

We now consider the parameters characterising the kinetics of water exchange on the dendritic Gd^{III} complexes of the three different generations and on the monomeric complex. From the data in Table 1, the general impression is that there are no dramatic differences from one generation to the other, nor between the monomer and the dendritic complexes. The water exchange rate constants at 298 K are essentially the same for all the four systems. It can be noted that k_{ex} for the generation 3 derivative, which has 96% of its terminal amine groups functionalized, is slightly smaller than for the monomer complex or for the higher generation dendrimer complexes, where the substitution is only 64% (gen. 4) and 53% (gen. 5). Although it cannot be excluded that the difference in the relative number of free amines affect the water exchange rate, it does not seem to be significant in our case. The positive activation volumes indicate a dissociatively activated mechanism, even if it is not as accentuated for the dendritic complex as it is for the monomer. All four complexes exhibit water exchange rate constants that are smaller than that found for $[Gd(DOTA)(H_2O)]^-$, which is the only monomeric macrocyclic Gd^{III}–poly(amino carboxylate) complex for which a water exchange rate has previously been determined. A comparison of all rate constants available (Table 2) shows that—within one class of gadolinium poly(amino carboxylate) complexes, linear or macrocyclic—substitution of one or two carboxylate arms by a functional group that coordinates less strongly towards the lanthanide ion (e.g., an amide or an alcoholic hydroxyl)[39] results in a decrease in the water exchange rate constant. The weaker coordination of these functional groups to the lanthanide ion compared to the carboxylate is well demonstrated by the smaller stability constants.[41–43] The main purpose of these modifications was to diminish the total charge on the complex, and thus the osmolality of the solution to be injected. Nowadays these functional groups provide a facile means to either dimerize the complexes or to attach them to polymers or antibodies. The first example for the decreased water exchange rate on substitution of carboxylate groups by amide functions was with $[Gd(DTPA\text{-}BMA)(H_2O)]$, where the k_{ex}^{298} is about one tenth of that of $[Gd(DTPA)(H_2O)]^{2-}$. The same trend (decrease in the rate constant by roughly a factor of five per substituted carboxylate group) is observed for three different dimeric complexes[17, 44] containing tetraazapolyacetate macrocycles as monomer units where one carboxylate arm of the DOTA is replaced by an alcoholic hydroxo group (e.g., in $[BO\{Gd(DO3A)(H_2O)\}_2]$), or an amide function (e.g. for $[piperazine\{Gd(DO3A)(H_2O)\}_2]$ and $[bisoxa\{Gd(DO3A)(H_2O)\}_2]$) ($BO(DO3A)_2$ = 2,11-dihydroxy-4,9-dioxa-1,12-bis-[(1,4,7,10-tetraaza-4,7,10-triacetato)cyclododecane]dodecane, $piperazine(DO3A)_2$ = bis-1,4,-(1-carboxymethyl-1,4,7,10-tetraaza-4,7,10-triacetate-cyclododecane)-1,4-diazacyclohexane, $bisoxa(DO3A)_2$ = bis-1,4,-(1-carboxymethyl-1,4,7,10-tetraaza-4,7,10-triacetatecyclododecane)-1,10-diaza-3,6-dioxadecane). The positive activation volumes show that water exchange is always dissociatively activated, that is, the exchange reaction passes through an eight-coordinate transition state or intermediate. An extensive ^{17}O NMR study on water exchange in a series of lanthanide(III) DTPA-BMA complexes has shown the importance of steric crowding in the inner coordination sphere on the rate in dissociatively activated exchange processes.[45] The more crowded the first sphere is, the less energy is needed to go from the nine-coordinate ground state to the eight-coordinate activated state, and the faster the dissociatively activated exchange reaction. The oxygen of an amide group is situated further away from the lanthanide ion than that of a carboxylate group.[46] Therefore, the coordination sphere is less crowded, and the consequence is a slower water exchange, as found for the monomeric $[Gd(DO3A\text{-}bz\text{-}NO_2)(H_2O)]$ as well as for the dimeric and dendritic complexes compared to $[Gd(DOTA)(H_2O)]^-$.

As one of the most important results of this study, we can conclude that the attachment of the macrocyclic unit to a large dendritic polymer molecule does not significantly influence the kinetics of water exchange on the complex. This finding makes it possible to predict water exchange rates for Gd complexes attached to dendrimers by determining this parameter for the monomeric chelate, before the tedious synthesis of the whole molecule.

The most important expectation for the dendritic contrast agents was that the rotational correlation time, τ_R, which limits the proton relaxivity of current contrast agents at imaging fields, would be increased. The longer the rotational correlation time, the greater is the potential for a higher relaxivity of the agent. In our present study as in previous ones,[6, 9, 17, 18, 25] we address the problem through the paramagnetic enhancement of the ^{17}O longitudinal relaxation [Eqs. (2) and (6–9)]. Therefore, τ_R is correlated with the value used for the effective distance between the gadolinium electron spin and the oxygen nuclear spin and with the values used for the quadrupolar coupling constant and its asymmetry parameter. However, since we use the same values for r, χ, and η in this and previous papers,[6, 9, 17, 18, 25] the comparison of the tumbling rates of the different gadolinium poly(amino carboxylates) is justified. As was expected for these large molecules, the values of τ_R are much higher than those found so far for monomeric or dimeric Gd^{III} complexes. However, it is surprising that the rotational correlation time of the linker chelate unit is considerably longer than that of $[Gd(DOTA)(H_2O)]^-$; it is comparable to dimeric complexes (Table 2). The bulky nitrobenzyl group seems to be large enough to efficiently slow down rotation. This is the first case where such a long rotational correlation time, as obtained from ^{17}O NMR measurements, is observed for a monomeric complex, since no data are known for other systems with similar large substituents. From proton relaxivity data Aime et al. determined a shorter rotational correlation time for a similar macrocyclic Gd^{III} complex.[47] The discrepancy generally found between the rotational correlation times, as obtained from ^{17}O and from proton relaxation data, is discussed in detail in another publication.[18] For the three dendrimers τ_R increases by approximately one fourth with each generation.

The overall tumbling rate and the intramolecular motion of dendrimers without any attached ligand has been the subject of several studies. By measuring the rotational correlation time of the carbon atoms in PAMAM-type dendrimers with ^{13}C NMR spectroscopy, Meltzer et al. found a striking difference between carbon atoms in the interior and on the surface: for internal carbons the correlation time increases by several orders of magnitude between generation 0 and 10, but it only doubles for carbon atoms on the surface.[22] According to this study, the rotational correlation time in the fifth generation of an amine-terminated PAMAM dendrimer is about 20 ps for the terminal carbons, while two types of interior C atoms are present having rotational correlation times of 80 and 800 ps, respectively (t = 25 °C). The τ_R^{298} obtained for G_5Gd_{52} from our ^{17}O measurements (870 ps) indicates that the attachment of the Gd^{III} chelate slows down the rotational movement of the amine terminus. The overall rotational correlation time of the dendritic complex in solution can be estimated from the hydrodynamic radius by using the Debye formula[48] [Eq. (15)], where r_{eff} is the effective radius, τ_R is the rotational correlation time of the

$$\tau_R = \frac{4\pi\eta r_{eff}^3}{3k_BT} \qquad (15)$$

0947-6539/96/0212-1612 $ 15.00+.25/0 Chem. Eur. J. 1996, 2, No. 12

molecule, and η is the viscosity of the solution. From the hydrodynamic radius measured in aqueous solution by light scattering,[10] a τ_R value of 5740 ps was obtained for G_5Gd_{52} from Equation (15). This method yields the highest limit for any rotational correlation time describing the tumbling of dendritic molecules. Figure 6 presents a comparison of the rotational correlation times determined by different methods: 1) ^{13}C relaxation for one type of internal and for terminal carbons in the generation 5 amine terminated PAMAM dendrimer,[22] 2) the hydrodynamic radius from the Debye formula for G_5Gd_{52}, and 3) calculated from ^{17}O NMR data for monomeric,[6, 9, 25] dimeric,[17, 18] and dendrimer Gd^{III} complexes. The Figure clearly shows that the flexibility of the applied macromolecules and the linker units must be eliminated to freeze out the rotation of the gadolinium–proton vector.

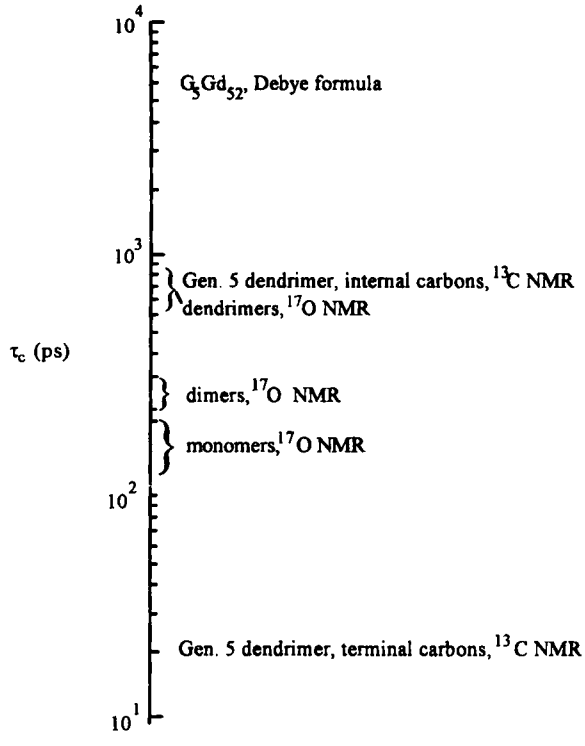

Fig. 6. The rotational correlation times as determined by ^{17}O NMR for monomeric, dimeric, and dendrimer Gd^{III} complexes; by ^{13}C T_1 measurements for an amine-terminated generation 5 PAMAM-type dendrimer [22], and from the molecular hydrodynamic diameter with the Debye formula for [G5(N{CS}N-bz-Gd{DO3A}-{H_2O})$_{52}$].

MRI contrast agents containing Gd^{III} are usually characterized by the magnetic field dependence of their proton relaxivity. Above approximately 20 MHz, proton relaxivity is limited by the rotation of the molecule. The NMRD (nuclear magnetic relaxation dispersion) profiles of contrast agents with long rotational correlation times show a characteristic high-field peak in this region, as observed for the dendritic complexes: $R_1 = 18.71$ and 14.57 $mM^{-1}s^{-1}$ for G_5Gd_{52} and G_3Gd_{23}, respectively, at 20 MHz (see Fig. 7a).[10] This high-field peak is not present in the profile of $[Gd(DOTA)(H_2O)]^-$.[49] The measured relaxivity is larger in the entire frequency range (0–50 MHz) for the higher generation complex G_5Gd_{52} than for G_3Gd_{23}. Given the relatively slow water exchange and the long rotational correlation time for these complexes, the following questions arise: How can even higher proton relaxivities be obtained? Is it solely the rotational correlation time that limits relaxivity? Previous simulations of the dependence of proton relaxivity on the water exchange rate and on the rotational correlation time have already indicated that, at very slow rotation, it will be the water exchange rate that limits relaxivity.[44] The longitudinal proton relaxivity is given by Equation (16),[5] where q is the number of

$$R_1 = \frac{q}{1000 \times 55.6}\,(T_{1m}^{H} + \tau_m^{H})^{-1} + R_{los} \qquad (16)$$

inner-sphere water molecules, T_{1m}^{H} is the proton relaxation rate in bound water, and τ_m^{H} is the residence time of protons in the inner-sphere; the total outer-sphere contribution is represented by R_{los}, and R_1 is expressed in units of $s^{-1}mM^{-1}$.

The τ_m for water molecules, as measured by ^{17}O NMR, gives only an upper limit for τ_m^{H}. That is, if proton exchange is more rapid than the exchange of water molecules, τ_m^{H} will be shorter than τ_m. However, the similarity of the values estimated from NMRD measured on solutions of Gd(DTPA–BMA)(H_2O) and by ^{17}O NMR confirms that τ_m^{H} is indeed equal to τ_m.[6, 7] This is because proton relaxation rates, and therefore the NMRD profiles, are only sensitive to exchange of protons regardless of whether protons exchange as individual entities or bound to entire water molecules. In order to visualize (see Fig. 7b) the effect of an increase in the water exchange rate on relaxivity for the dendrimers, we computed the proton relaxivity at 20 MHz and 37 °C as a function of the proton exchange rate for the two dendrimer complexes G_5Gd_{52} and G_3Gd_{23} as well as for $[Gd(DOTA)(H_2O)]^-$, using Equation (16). The same outer-sphere contribution (that of $[Gd(DOTA)(H_2O)]^-$) was used throughout; this can only lead to negligible deviations in the calculated relaxivity of the dendritic complexes, because the inner-sphere contribution is so much greater. The value of the bound water relaxation time T_{1m}^{H} is given by Equation (17),

$$\frac{1}{T_{1m}^{H}} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\hbar^2\gamma_I^2\gamma_s^2}{r^6} S(S+1)\left[\frac{3\tau_{d1}}{\omega_I^2\tau_{d1}^2} + \frac{7\tau_{d2}}{\omega_s^2\tau_{d2}^2}\right] \qquad (17)$$

where $1/\tau_{di} = 1/\tau_R + 1/T_{ie} + 1/\tau_m = 1/\tau_R + 1/T_{ie}$ for present purposes because our measurements show that τ_m is much greater than τ_R, and therefore T_{1m}^{H} is independent of τ_m.

For $[Gd(DOTA)(H_2O)]^-$ an increase in the water exchange rate would not result in an increased proton relaxivity at 20 MHz according to Equation (16), because τ_m is already shorter than T_{1m}^{H}, and any further increase in the exchange rate is of no consequence. However, increasing τ_R for $[Gd(DOTA)(H_2O)]^-$ would result in a high-field boost in proton relaxivity[44] according to Equation (17), as previously stated. For the generation 5 dendrimer complex, τ_R increases by about a factor of 8 relative to the value for $[Gd(DOTA)(H_2O)]^-$, and we would therefore expect the relaxivity to also increase by this factor. Experimentally, the relaxivity increases by only a factor of 5, and the differences between the expected and measured relaxivities must be due to the fact that τ_m becomes the limiting value in Equation (16), because of the decrease in T_{1m}^{H} brought about by the increase in τ_R. As shown in Figure 7b, a tenfold increase in the exchange rate for the dendrimers would increase proton relaxivity by almost a factor of two. These dendrimers represent an example of potential MRI contrast agents where the proton relaxivity at physiological temperatures, though high, does not reach its potential value because it is limited by the water exchange rate.

These findings have important implications for future development. They indicate that high molecular weight complexes like these dendrimers have rotational correlation times that are long enough for the water exchange to influence the overall

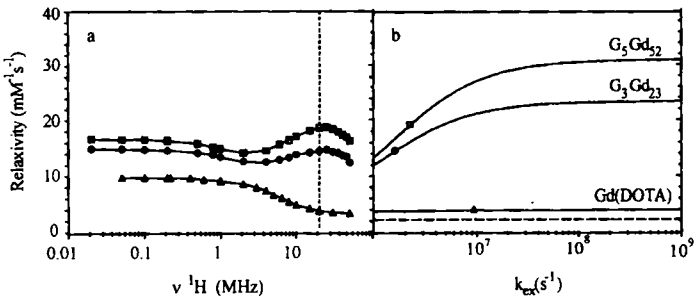

Fig. 7. a) NMRD profiles of [G5(N{CS}N-bz-Gd{DO3A}{H_2O})$_{52}$] (□), [G3(N-{CS}N-bz-Gd{DO3A}{H_2O})$_{23}$] (○) (in water, 37 °C), and [Gd(DOTA)(H_2O)]$^-$ (△) (in saline buffer, 39 °C). The vertical dashed line indicates the 20 MHz proton frequency. b) Dependence of proton relaxivity on the water exchange rate as calculated with parameters for [G5(N{CS}N-bz-Gd{DO3A}{H_2O})$_{52}$], [G3(N{CS}N-bz-Gd{DO3A} {H_2O})$_{23}$], and [Gd(DOTA)(H_2O)]$^-$, at 20 MHz proton frequency (see text). The symbols represent the actual proton relaxivities measured for [G5(N{CS}N-bz-Gd{DO3A}{H_2O})$_{52}$] (□), [G3(N{CS}N-bz-Gd{DO3A}-{H_2O})$_{23}$] (○), and [Gd(DOTA)(H_2O)]$^-$ (△). The dashed line represents the outer-sphere contribution.

relaxivity. Therefore, beside slowing down rotation, a further improvement in the efficiency of the contrast agents can be achieved with higher water exchange rates. This may be possible by changes in the coordinating groups of the chelate so as to facilitate the dissociative activation step.

Experimental Procedure

Sample Preparation: The PAMAM dendrimers with an ammonia core were prepared as previously described by Tomalia. The complexes [G5(N{CS}N-bz-Gd{DO3A}{H_2O})$_{52}$] (G_5Gd_{52}), [G4(N{CS}N-bz-Gd{DO3A}{H_2O})$_{30}$] (G_4-Gd_{30}), and [G3(N{CS}N-bz-Gd {DO3A}{H_2O})$_{23}$] (G_3Gd_{23}), and the free ligands [G5(N{CS}N-bz-DO3A)] and DO3A-bz-NO_2 were synthesized and provided by Nycomed, USA [10], and used without further purification. The dendrimers contain 24 (gen. 3), 48 (gen. 4), and 96 (gen. 5) amine groups on the surface, from which 23, 30, and 52 are functionalized with the macrocyclic complex for the generation 3, 4, and 5 dendrimers, respectively. These represent the maximum numbers of substituted terminal amine groups that could be obtained by 20–30% molar excess of DO3A-bz-NCS per amine [10]. For preparing the complex [Gd(DO3A-bz-NO_2)(H_2O)], a $Gd(ClO_4)_3$ stock solution was prepared by dissolving Gd_2O_3 (Nucor Corp., 99.99%) in a slight excess of $HClO_4$ (Merck, p.a., 70%) in doubly distilled water, followed by filtration. The concentration of the solution was determined by titration with Na_2H_2EDTA solution using xylenol orange as indicator. A weighed quantity of the solid ligand DO3A-bz-NO_2 was dissolved in a solution containing a known quantity of NaOH for stochiometric deprotonation. To this solution an appropriate amount of the $Gd(ClO_4)_3$ stock solution was added under stirring and heating for two hours (40 °C). The paramagnetic solutions of the dendrimer complexes for the ^{17}O NMR study were prepared by weight of solid complex. All samples for the ^{17}O NMR measurements contained 2% of enriched water $H_2{}^{17}O$ (Yeda, Israel). The pH was adjusted by adding weighed amounts of 0.1 M perchloric acid and sodium hydroxide solution and was measured with a combined glass electrode, calibrated with standard Methrom buffers of pH = 4.00 and 7.00. The absence of free Gd^{3+} in the solutions was verified by using xylenol orange indicator [50]. Variable-temperature ^{17}O NMR measurements on G_3Gd_{23} at $B = 14.1$ T were carried out at two different Gd^{III} concentrations to exclude artifacts of concentration effects. For the UV/Visible spectrophotometric measurements a solution of the complex [G5(N{CS}N-bz-Eu{DO3A}{H_2O})$_{52}$] was prepared in a similar way as described above for the [Gd(DO3A-bz-NO_2)(H_2O)]). The pH of the solution was adjusted and the absence of free Eu^{3+} was tested as for the ^{17}O NMR samples. The compositions of all the solutions are given in Table 3.

^{17}O NMR Measurements: Variable temperature ^{17}O NMR measurements were performed using Bruker spectrometers (AMX2-600: 14.1 T, 81.4 MHz; AM-400: 9.4 T, 54.2 MHz; and a 1.41 T, 8.14 MHz electromagnet connected to a AC-200 console). Bruker VT-1000 and VT-2000 temperature control units were used to stabilize the temperature, which was measured by a substitution technique [51]. The samples were sealed in glass spheres and fitted into 10 mm NMR tubes, in order to eliminate susceptibility corrections to the chemical shift [52]. 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 [53] or, for line widths greater than 500 Hz, directly from the line widths themselves. Variable-pressure NMR measurements up to 200 MPa were performed on a Bruker AM-400 spectrometer equipped with a home-built high-pressure probe head [54]. The temperature was controlled by circulating fluid from a temperature bath, and was measured by means of a built-in Pt resistor. The relaxation rates and, for the variable-temperature measurement, the chemical shifts were measured for the paramagnetic solutions and for a reference solution (perchloric acid containing 1% ^{17}O; pH = 3.4); thus, the diamagnetic shift was neglected. Since the dendrimer solutions were rather dilute (more dilute than the monomeric and dimeric Gd^{III}-complex solutions used in the former ^{17}O NMR studies [6,9,17,18,25,45]), the acidified water as reference material is appropriate. This is also supported by the concentration invariance of the ^{17}O NMR results.

Table 3. Compositions of the aqueous solutions used in the variable-temperature (VT) and variable-pressure (VP) ^{17}O NMR and variable-temperature UV/Vis spectrometry measurements.

No.	Solution	[Ln^{3+}] (mol kg^{-1})	$10^3 P_m$	pH
1	acidified water			3.40
2	[G5(N{CS}N-bz-Gd{DO3A}{H_2O})$_{52}$] (VT)	0.0226	0.409	5.90
3	[G4(N{CS}N-bz-Gd{DO3A}{H_2O})$_{30}$] (VT)	0.0332	0.601	6.25
4	[G3(N{CS}N-bz-Gd{DO3A}{H_2O})$_{23}$] (VT and VP)	0.0996	1.795	5.73
5	[G3(N{CS}N-bz-Gd{DO3A}{H_2O})$_{23}$] (VT)	0.1706	3.070	5.97
6	[Gd(DO3A-bz-NO_2)(H_2O)] (VT and VP)	0.0332	0.597	4.04
7	[G5(N{CS}N-bz-Eu{DO3A}{H_2O})$_{52}$] (UV/Vis)	0.0221		5.90

UV/Vis Spectrophotometry: A variable-temperature UV/Vis study was performed to check the presence of coordination equilibria for the complexes studied [55]. For this purpose we used the Eu^{III} complex, which has a very sensitive electronic absorption in the range of $578 < \lambda < 581$ nm. The measurements were done on a double-beam Perkin-Elmer Lambda 19 spectrometer in thermostatizable cells with a 10 cm optical pathlength. The transitions were measured at 30 and 90 °C. A solution of the free ligand of the same concentration as the corresponding Eu^{III} complex was used as reference.

Data analysis: The simultaneous least-squares fitting was performed by the program Scientist® for Windows™ by Micromath®, version 2.0. The reported errors correspond to one standard deviation obtained by the statistical analysis and may be larger due to possible systematic errors.

Acknowledgements: We thank Larry Margerum and Brian Campion at Nycomed Salutar for providing the dendrimers used in this study. We also thank Kenneth E. Kellar for useful discussions and advice, and Nycomed Inc. for their scientific and financial support. This work was also supported by the Swiss National Science Foundation (Grant No. 39483.93) and the Swiss OFES as part of the European COST D1 action.

Supplementary Material Available: The following material may be obtained from the correspondence author: variable-temperature ^{17}O transverse and longitudinal relaxation rates and chemical shifts of [G5(N{CS}N-bz-Gd{DO3A}{H_2O})$_{52}$], [G4(N{CS}N-bz-Gd{DO3A}{H_2O})$_{30}$], [G3(N{CS}N-bz-Gd{DO3A}{H_2O})$_{23}$], and [Gd(DO3A-bz-NO_2)(H_2O)] solutions and of acidified water at 1.4, 9.4, and 14.1 T (Tables S1–12); variable-pressure ^{17}O transverse relaxation rates of [G3(N{CS}N-bz-Gd{DO3A}{H_2O})$_{23}$] and [Gd(DO3A-bz-NO_2)(H_2O)] solutions at 9.4 T (Tables S13–14) (9 pages).

Received: July 29, 1996 [F424]

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