Phosphinic derivative of DTPA conjugated to a G5 PAMAM dendrimer: an ^{17}O and ^{1}H relaxation study of its Gd(III) complex†

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A DTPA-based chelate containing one phosphinate group was conjugated to a generation 5 polyamidoamine (PAMAM) dendrimer *via* a benzylthiourea linkage. The Gd(III) complex of this novel conjugate has potential as a contrast agent for magnetic resonance imaging (MRI). The chelates bind Gd^{3+} *via* three nitrogen atoms, four carboxylates and one phosphinate oxygen, and one water molecule completes the inner coordination sphere. The monomer Gd(III) chelates bearing nitrobenzyl and aminobenzyl groups ($[Gd(DTTAP\text{-}bz\text{-}NO_2)(H_2O)]^{2-}$ and $[Gd(DTTAP\text{-}bz\text{-}NH_2)(H_2O)]^{2-}$) as well as the dendrimeric Gd(III) complex G5-$(Gd(DTTAP))_{63}$) were studied by multiple-field, variable temperature ^{17}O and ^{1}H NMR. The rate of water exchange is faster than that of $[Gd(DTPA)(H_2O)]^{2-}$ and very similar on the two monomeric complexes (8.9 and 8.3 × 10^6 s^{-1} for $[Gd(DTTAP\text{-}bz\text{-}NO_2)(H_2O)]^{2-}$ and $[Gd(DTTAP\text{-}bz\text{-}NH_2)(H_2O)]^{2-}$, respectively), while it is decreased on the dendrimeric conjugate (5.0 × 10^6 s^{-1}). The Gd(III) complex of the dendrimer conjugate has a relaxivity of 26.8 mM^{-1} s^{-1} at 37 °C and 0.47 T (corresponding to ^{1}H Larmor frequency of 20 MHz). Given the contribution of the second sphere water molecules to the overall relaxivity, this value is slightly higher than those reported for similar size dendrimers. The experimental ^{17}O and ^{1}H NMR data were fitted to the Solomon–Bloembergen–Morgan equations extended with a contribution from second coordination sphere water molecules. The rotational dynamics of the dendrimeric conjugate was described in terms of global and local motions with the Lipari–Szabo approach.

Introduction

Paramagnetic metal complexes are widely used as contrast agents for magnetic resonance imaging (MRI). They increase the relaxation rate of the water proton spins in their surroundings, which enhances the image contrast. Gd(III) is often applied as the paramagnetic metal ion for its electronic properties (the seven unpaired electrons make it highly paramagnetic, and its electronic relaxation is relatively slow due to the ground state *S* symmetry).[1]

The term relaxivity (r_1) defines the enhancement of the relaxation rate of the water protons per mmol of Gd^{3+} and it is directly proportional to the efficiency of the contrast agent. The relaxivity depends on several parameters, such as the residence time of the inner sphere water molecule (τ_M), the reorientation time of the complex (τ_R) and the electronic relaxation times (T_{1e} and T_{2e}). By fine-tuning these parameters, one can obtain a contrast agent of very high efficiency. Since little is known of the relation between molecular structure and electronic spin relaxation, the design of new contrast agents is mainly focused on the optimisation of τ_M and τ_R.

The exchange of the first coordination sphere water is generally too slow on currently used contrast agents.[1] It could be accelerated by introducing a bulky phosphinic or phosphonic group instead of a carboxylate:[2–4] the increased steric crowding and negative charge brought by the phosphorus-containing moiety forces the bound water molecule to leave faster. The phosphonic and phosphinic functions can also induce hydrogen bond formation, which will prolong the residence time of the outer sphere molecules in the proximity of the paramagnetic centre and therefore influence their relaxation times.[5,6] Those water molecules constitute the so-called second hydration sphere.[7] The rotation of the paramagnetic complex can be slowed down by increasing the molecular volume and maintaining a rigid internal structure. This can be achieved by self-assembly of small chelates (like micelle formation), or by covalent or non-covalent conjugation to macromolecules, such as peptides, polysaccharides or dendrimers.[1,8–12]

PAMAM (polyamidoamine) dendrimers are water soluble, spheroidal particles, composed of repeated polyamidoamine units and primary amino groups on the surface, which can be derivatised. The generation 5 dendrimer, used in this work, contains 128 primary amino groups.[13,14] An example of a previously studied phosphinic chelate conjugated to PAMAM dendrimer is the G2-$(Gd(DO3A\text{-}P^{ABn}))_{16}$ complex[3] which shows high relaxivity

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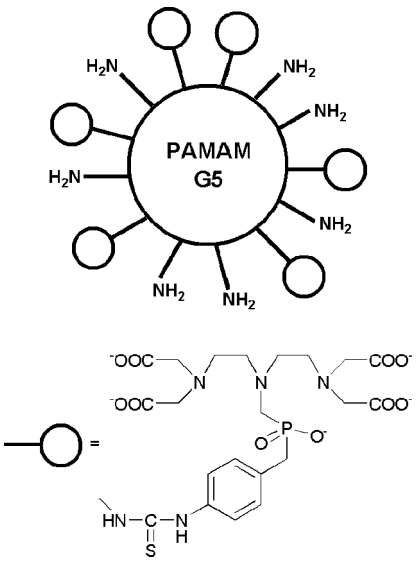
† Electronic supplementary information (ESI) available: Equations used for the analyses of ^{17}O NMR and ^{1}H NMRD data; tables of the variable temperature ^{17}O NMR and ^{1}H NMRD relaxation data and figures of ^{17}O NMR chemical shift data; calculation of the number of chelating groups per molecule of dendrimer and a full list of parameters for the fitting of $[Gd(DTTAP\text{-}bz\text{-}NO_2)(H_2O)]^{2-}$, $[Gd(DTTAP\text{-}bz\text{-}NH_2)(H_2O)]^{2-}$ and G5-$(Gd(DTTAP))_{63}$. See DOI: 10.1039/b517847a

(14.1 mM^{-1} s^{-1}, 37 °C, 20 MHz) due to fast water exchange and a second sphere contribution. A phosphinate containing DTPA derivative, DTTAP-bz-NH_2, was conjugated to inuline and a relaxivity of 18.3 mM^{-1} s^{-1} was reported for its Gd(III) complex (37 °C, 0.47 T).[15] This relaxivity was lower than expected according to the molecular mass of the conjugate, probably due to the internal flexibility of the macromolecule.



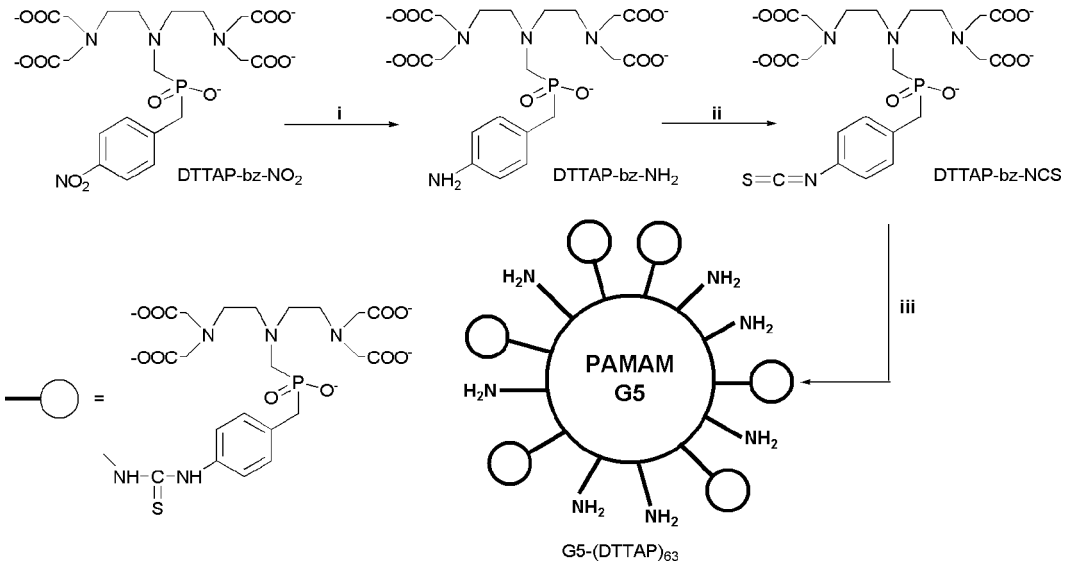
Scheme 1 Structure of the dendritic ligand.

In this paper, we report the conjugation of the same monomeric chelate DTTAP-bz-NH_2 to a generation 5 PAMAM dendrimer (Schemes 1 and 2), which has a greater molecular weight and should also be more rigid than inulin, and therefore a more significant increase in relaxivity is expected for the Gd(III) complex. The monomeric chelates $[Gd(DTTAP\text{-}bz\text{-}NO_2)(H_2O)]^{2-}$ and $[Gd(DTTAP\text{-}bz\text{-}NH_2)(H_2O)]^{2-}$ as well as the dendrimeric conjugate [PAMAM-G5(N{CS}N-bz-Gd{DTTAP}{H_2O}$^{2-}$)$_{63}$] (later in the text referred to as G5-(Gd(DTTAP))$_{63}$) were evaluated using variable temperature multiple-field NMR spectroscopy and the experimental data were fitted to the commonly used equations based on the Solomon–Bloembergen–Morgan approximation,[1] extended by a second sphere contribution to the overall relaxivity. The experimental ^{1}H and ^{17}O longitudinal relaxation rates obtained for the dendrimer were evaluated using the Lipari–Szabo approach yielding local and global rotational correlation times.[9,16,17] All the ligands discussed are depicted in Scheme 3.

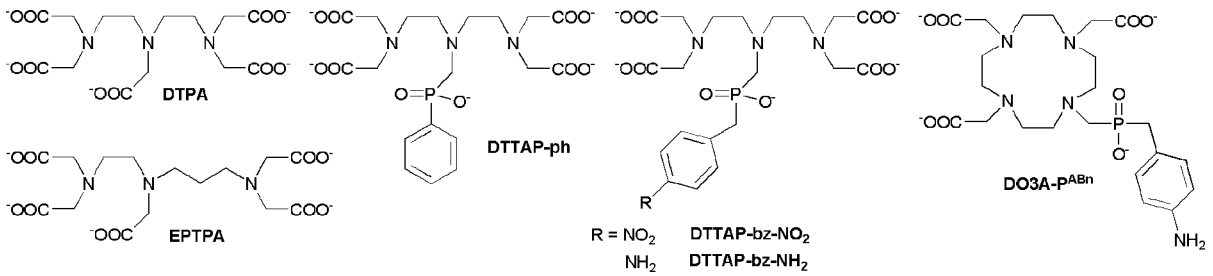
Experimental

Materials and methods

All reagents and solvents were commercially available. The ethylenediamine core PAMAM G5 dendrimer with primary amines on the surface was purchased as a 5.26 w/w% aqueous



Scheme 2 Synthesis of the dendrimeric chelate; (i) H_2/Pd/C, (ii) $CSCl_2$, CCl_4, HCl, (iii) PAMAM G5, 1 M LiOH.



Scheme 3 Structures of monomeric chelates discussed in the text.

solution from Dendritech Inc. (Midland, MI). MILIPORE ultrafiltration membranes YM-10 were obtained from Amicon (Centriprep®, regenerated cellulose 10 000 MWCO, Bedford, MA).

The luminescence measurements were performed on an Aminco ABS2 spectrofluorimeter. Luminescence decay were obtained at 615 nm after excitation at 395 nm.

^{1}H NMRD profiles were performed on a Stelar Spinmaster FFC fast field cycling relaxometer covering magnetic fields from 2.35×10^{-4} T to 0.47 T (proton Larmor frequency range 0.01–20 MHz). The temperature was fixed by a gas flow and controlled by a VTC90 temperature control unit. At higher fields, relaxivities were measured on Bruker Minispecs mq30 (30 MHz), mq40 (40 MHz) and mq60 (60 MHz) and on Bruker 100 MHz (2.35 T) and 200 MHz (4.7 T) cryomagnets connected to a Bruker Avance-200 console and on a Bruker DRX 400 (400 MHz, 9.4 T). In each case, the temperature was measured by the substitution technique.[18]

Longitudinal and transverse ^{17}O relaxation rates were measured at temperatures from 279 K to 359 K. The data were recorded on Bruker ARX 400 (9.4 T, 54.2 MHz) and Bruker Avance-200 (4.7 T, 27.1 MHz) spectrometers. VT 3000 temperature control units were used to maintain a constant temperature, which was measured by a substitution technique.[18] The samples were sealed in glass spheres, fitting into 10 mm NMR tubes. Longitudinal relaxation rates, $1/T_1$, were obtained by the inversion recovery method while transverse relaxation rates, $1/T_2$, were measured by the Carl–Purcell–Meiboom–Gill spin-echo technique. As external references, acidified water (pH 3.3) was used for the monomeric chelates and the Y(III) complex (of a concentration and pH identical to those of the Gd(III) complex) for the dendrimer.

Elemental analysis was performed by Solvias AG, Basel, Switzerland. Analysis of the ^{17}O NMR and ^{1}H NMRD experimental data was performed with the Visualiseur/Optimiseur programs on a Matlab platform version 6.5.[19]

Diethylenetriamine-*N*′-methylene(*p*-aminobenzyl)-phosphinic-*N*,*N*,*N*″,*N*″-tetraacetic acid (DTTAP-bz-NH$_2$)

The synthesis of DTTAP-bz-NO$_2$ and DTTAP-bz-NH$_2$ was performed according to the literature.[15] The purification of DTTAP-bz-NH$_2$ was improved from the original procedure, performing a chromatography on a SiO$_2$ column (^{i}PrOH : NH$_3$: H$_2$O 10 : 3 : 3). R_f (DTTAP-bz-NH$_2$) = 0.33. The cations were removed on a column of strong anion exchanger (AG 1-X4, 100–200 mesh, Bio-Rad, AcO$^-$ form, 130 ml). After washing the column with water, the product was eluted using HCl solution (5%). The solvents were evaporated under reduced pressure into the resulting yellow oil. All the amount obtained was used for the next reaction.

MS (ESI) for DTTAP-bz-NO$_2$, *m*/*z* (%): 549.4 [M + H$^+$], calcd. for $C_{20}H_{29}N_4O_{12}P$: $M = 548.44$

MS (ESI) for DTTAP-bz-NH$_2$, *m*/*z* (%): 519.3 [M + H$^+$], calcd. for $C_{20}H_{31}N_4O_{10}P$: $M = 518.45$

Diethylenetriamine-*N*′-methylene(*p*-isothiocyanatobenzyl)-phosphinic-*N*,*N*,*N*″,*N*″-tetraacetic acid (DTTAP-bz-NCS)

425 mg (0.64 mmol) of DTTAP-bz-NH$_2$·4HCl was dissolved in HCl (7.3 ml, 3M). Carbon tetrachloride (4.2 ml, 44 mmol) and thiophosgene (2.0 ml, 26 mmol) were added and the reaction mixture was vigorously stirred at RT for 6 h. The completion of the reaction was checked using TLC (^{i}PrOH : NH$_3$: H$_2$O 7 : 3 : 3, R_f = 0.54, the spot remained uncoloured under ninhydrin detection). All the solvents were evaporated and the solid product was dried overnight under vacuum at 45 °C to yield DTTAP-bz-NCS which was used without further purification for the next reaction.

NMR characterization was in agreement with the previously published data of this compound.[15]

MS (ESI), *m*/*z* (%): 561.3 [M + H$^+$], calcd. for $C_{21}H_{29}N_4O_{10}SP$: $M = 560.515$

PAMAM G5-(DTTAP)$_{63}$

DTTAP-bz-NCS obtained above was dissolved in H$_2$O (8.5 ml) with the addition of LiOH (3.8 ml, 1 M) until everything dissolved. The resulting pH was 7. The aqueous solution of PAMAM G5 dendrimer (2.347 g, 5.26 w/w% in water, 0.5482 mmol of NH$_2$ groups) was added dropwise to the reaction mixture. The pH was increased to a value of 8–9 and the reaction mixture was stirred at 30 °C for 5 days. The pH was maintained constant by addition of LiOH (1 M).

The reaction mixture was then filtered through a filter paper and then through a 22 μm disposable filter. Free chelate was removed by ultrafiltration (Centriprep® YM-10) using centrifuge (3800 rpm). The resulting yellow solution was evaporated and vacuum dried overnight at 45 °C to yield 342 mg of a yellow solid.

Assessment of Gd(III) loading on the dendrimer

The number of chelates linked to the dendrimer was assessed by ^{1}H NMR, by complexometric titration using GdCl$_3$[20] and by elemental analysis. The integration of the ^{1}H NMR spectrum (aromatic *vs.* aliphatic regions) gave an estimation of 48% loading of the total number of primary amino groups.

The complexometric titration allows the determination of the number of chelating units per mass of the product. A solution of a known amount of the dendrimeric conjugate was titrated by a GdCl$_3$ solution (buffered with urotropin/HCl, pH 5.8, xylenol orange indicator). The complexometric titration in combination with the elemental analysis allowed calculating the average loading of the dendrimer which was found to be 63 molecules of chelate per 128 amino groups on one molecule of dendrimer (49%). Details are given in the ESI†.

Elemental analysis calcd. (%) for $C_{1262}H_{2528}N_{506}O_{252}$·63 $C_{21}H_{29}N_4O_{10}PS$ (64137.32): C 48.4, H 6.84, O 22.0, N 16.6, P 3.04, S 3.15; found: C 48.8, H 7.05, O 21.2, N 17.3, P 2.71, S 2.81.

Sample preparation

Gadolinium(III) and yttrium(III) complexes for ^{17}O NMR and ^{1}H NMRD measurements were prepared by mixing a slight excess of the ligands (5%) with metal chloride solutions. GdCl$_3$ and YCl$_3$ solutions were prepared by dissolving metal oxides in slight excess of HCl (Merck, p.a., 25%) followed by evaporation of solvents and dissolution in double distilled water. The concentration of the stock solutions was determined by titration with Na$_2$H$_2$EDTA solution (indicator xylenol orange). The pH of the complex solutions was adjusted using NaOH (1 M) and HCl (1 M). The absence of the free metal ion was checked with xylenol orange

indicator at pH 5.8. The samples used for ^{17}O NMR were enriched to 1% with ^{17}O-enriched water (Isotrade GmbH).

Results and discussion

Synthesis of the dendrimeric conjugate

The conjugation of the chelate to the PAMAM G5 dendrimer was performed *via* thiourea linkage according to Scheme 2. The NCS-chelate was used in 20% molar excess and the loading ran to 49% of the total possible substitution places. Even on addition of a novel portion of 200% excess of the DTTAP-bz-NCS, no higher loading of the dendrimer was achieved. For carboxylate derivatives, higher loadings have usually been reported.[20] One possible explanation could be that the phosphinate groups form stronger hydrogen bonds with the remaining unsubstituted amines than the carboxylates and this disables further conjugation. Nevertheless, the non-complete substitution of the dendrimer can be an advantage as it leaves space for further modification and functionalization of the primary amines. For instance, one can attach any targeting group to deliver the agent to a desired place in the body, or an antenna that can act as a reporter in another imaging modality (dyes for near infrared imaging, radioactive tracers for SPECT, *etc.*) giving rise to 'multimodal' contrast agents.

^{17}O NMR and ^{1}H relaxivity measurements on the monomers [Gd(DTTAP-bz-NO_2)$(H_2O)]^{2-}$ and [Gd(DTTAP-bz-NH_2)$(H_2O)]^{2-}$

Variable temperature ^{17}O NMR measurements were performed at 9.4 T (54.2 MHz) on aqueous solutions of the chelates and were referenced to an acidified water solution (aqueous $HClO_4$, pH 3.3). The data obtained for [Gd(DTTAP-bz-NO_2)$(H_2O)]^{2-}$ and [Gd(DTTAP-bz-NH_2)$(H_2O)]^{2-}$ are very similar (Fig. 1A, 1B, 2A, 2B). The temperature dependence of the transverse ^{17}O relaxation rates indicates relatively fast water exchange (the maximum of the $\ln(1/T_{2r})$ *versus* $1/T$ curve (Fig. 1A, 1B) is shifted towards low temperatures with respect to analogous data[21] for [Gd(DTPA)$(H_2O)]^{2-}$).

By analogy to similar Gd(III) complexes,[2] coordination of one inner-sphere water molecule was expected. It was confirmed by the luminescence decay measurement method. The value of luminescence lifetime measured for the [Eu(DTTAP-bz-NH_2)$(H_2O)]^{2-}$ complex ($\tau = 582$ μs) corresponds with the hydration number $q = 1$ according to well-proved relationships.[22,23]

Reduced ^{17}O chemical shifts were also measured for these two gadolinium complexes (data available in the ESI†), however, they were not included in the calculations since the values are systematically smaller than what is expected for a chelate with one coordinated water molecule. A possible explanation could be the influence of the second sphere water molecules. As it has been shown by quantum chemical calculations, the sign of spin density on Gd(III) complexes can depend on spatial arrangement and therefore second sphere ^{17}O chemical shifts could have a different sign compared to the first sphere shift.[24]

Proton relaxivities were measured as a function of the ^{1}H Larmor frequency at four temperatures (5, 25, 37 and 50 °C) and at a pH close to physiological (pH ~ 6.5). The shape of the NMRD curves and their temperature dependence

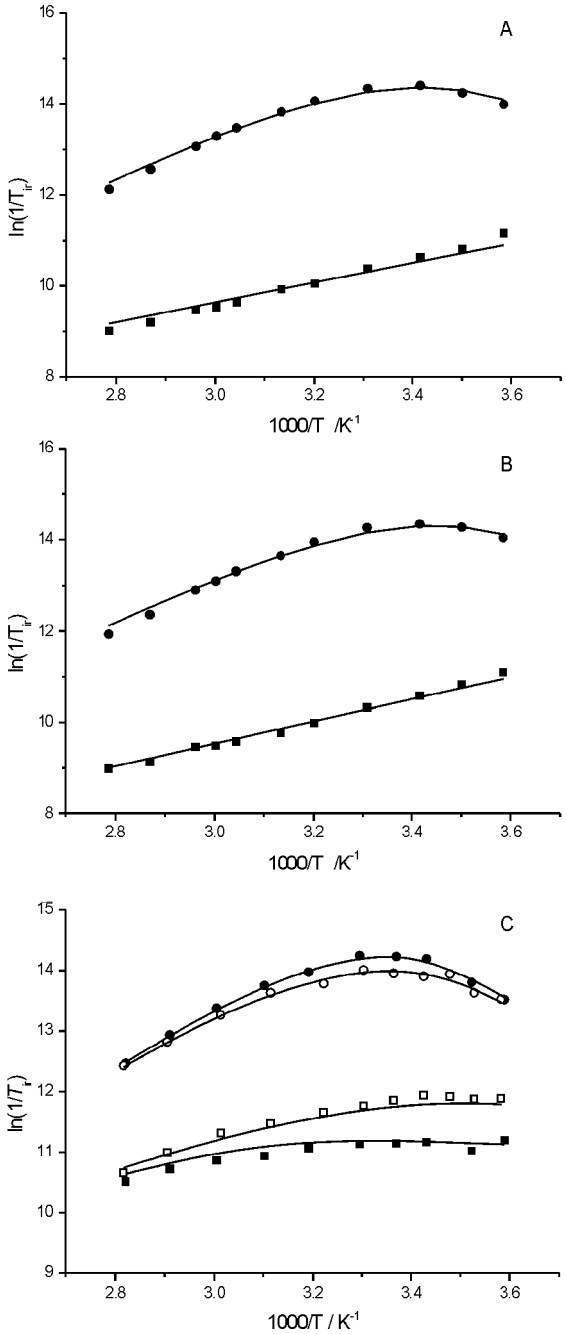


Fig. 1 Reduced longitudinal (■) and transverse (●) relaxation rates of: (A) [Gd(DTTAP-bz-NO_2)$(H_2O)]^{2-}$; (B) [Gd(DTTAP-bz-NH_2)$(H_2O)]^{2-}$; (C) G5-(Gd(DTTAP))$_{63}$ (4.7 T –□, ○; 9.4 T –■, ●); the lines are calculated using Solomon–Bloembergen–Morgan theory (A, B) and the Lipari–Szabo approach (C).

(r_1 decreases with increasing temperature) follows the general trend for small molecular weight complexes (Fig. 2A, 2B). The r_1 values of [Gd(DTTAP-bz-NO_2)$(H_2O)]^{2-}$ and [Gd(DTTAP-bz-NH_2)$(H_2O)]^{2-}$ are slightly higher than those of currently used contrast agents (5.24 and 5.02 mM^{-1} s^{-1} for [Gd(DTTAP-bz-NO_2)$(H_2O)]^{2-}$ and [Gd(DTTAP-bz-NH_2)$(H_2O)]^{2-}$, respectively, *vs.* 4.02 mM^{-1} s^{-1} for [Gd(DTPA)$(H_2O)]^{2-}$) but are very similar to the phosphorus containing analogue [Gd(DTTAP-ph)$(H_2O)]^{2-}$

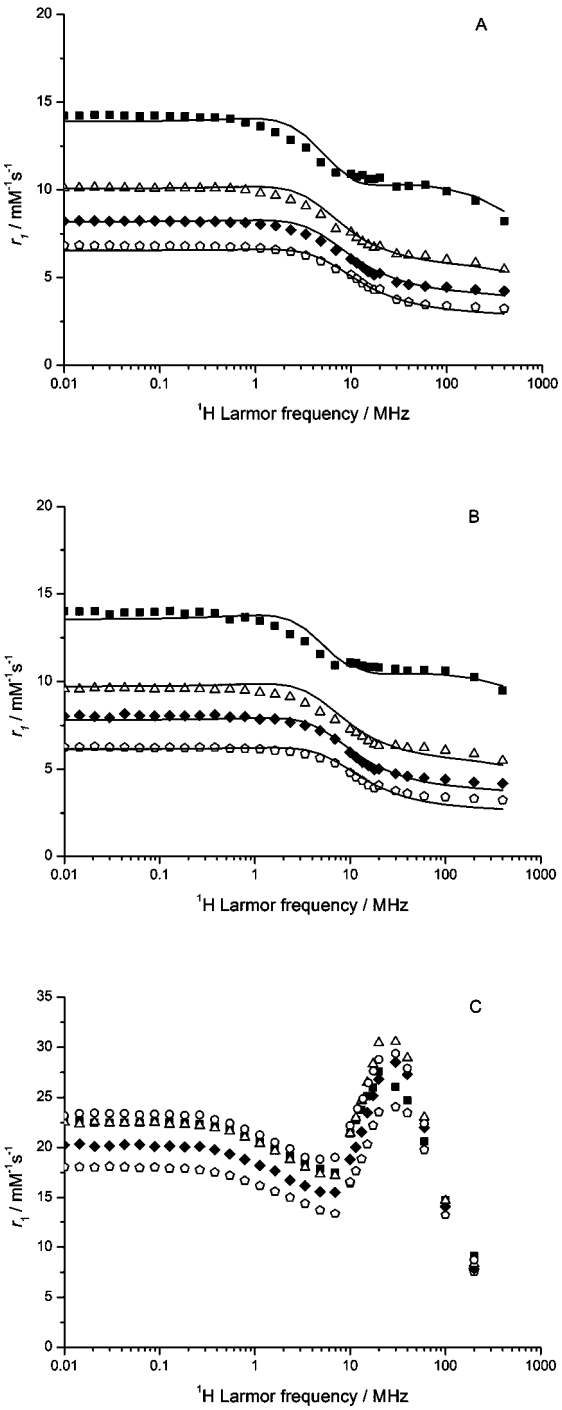


Fig. 2 ^{1}H NMRD profiles: (A) [Gd(DTTAP-bz-NO_2)$(H_2O)]^{2-}$; (B) [Gd(DTTAP-bz-NH_2)$(H_2O)]^{2-}$; (C) G5-(Gd(DTTAP))$_{63}$ (■ 5 °C, ○15 °C, △ 25 °C, ◆ 37 °C and ○ 50 °C; the lines are calculated using Solomon–Bloembergen–Morgan theory).

complex[2] (Scheme 3, Table 1). This originates from the contribution of second sphere water molecules, present in the phosphinate containing complexes.

The relaxation data obtained by the variable temperature ^{17}O NMR and ^{1}H NMRD measurements were analysed simultaneously (Fig. 1A with 2A, Fig. 1B with 2B for [Gd(DTTAP-bz-NO_2)$(H_2O)]^{2-}$ and [Gd(DTTAP-bz-NH_2) $(H_2O)]^{2-}$, respectively). To obtain the best least-square fits, a second sphere water coordination shell had to be taken into account in the set of equations.[2] This term was necessary not only to describe proton relaxation, but also longitudinal oxygen-17 relaxation. Longitudinal ^{17}O relaxation is influenced by dipolar and quadrupolar contributions, both related to τ_R. The second sphere water feels the rotation of the chelate, consequently the T_1 values measured on bulk water will be affected by the presence of second sphere water molecules. On the other hand, ^{17}O T_2 relaxation is mainly driven by the scalar contribution thus it is not sensitive to τ_R and consequently to the presence of a second hydration shell.

The total number of parameters used for the simultaneous fitting was 23; several of them are common to both sets of ^{17}O and ^{1}H experimental data. As discussed above and similarly to the previously reported inulin-(Gd(DTTAP))$_{23}$,[15] the number of inner sphere water molecules was fixed to one. The number of second sphere water molecules was fixed to 2, based on analogous phosphinates studied by Kotek *et al.*[2] The distances between oxygen/proton of the coordinated water and gadolinium were fixed to usual values:[1] 2.5 Å and 3.1 Å, respectively. The distance between the outer sphere water proton and the gadolinium was fixed to 3.5 Å. To describe the contribution of the second sphere water, four additional parameters were included: the average residence time of the second sphere water ($\tau_{M_{2nd}}^{298}$) was fixed to 50 ps, based on the simulations of Borel *et al.*[25] Its activation enthalpy ($\Delta H_{2nd}^{\ddagger}$) was set to 35 kJ mol^{-1}, as it was found for similar molecules by Kotek *et al.*,[2] but the quality of the fit was not significantly influenced upon changing this parameter between 15–50 kJ mol^{-1}. The distances of the second sphere oxygen and proton from the gadolinium ion were fixed to 4.1 Å and 3.5 Å, respectively. For the quadrupolar coupling constant $\chi(1 + \eta^2/3)^{1/2}$ we used the value for pure water (7.58 MHz).[21] The value of the hyperfine coupling constant ($A/\hbar$), was fixed to -3.8×10^6 rad s^{-1}, as previously obtained for the [Gd(DTPA)$(H_2O)]^{2-}$ complex.[21] The activation energy of τ_V, E_v, had to be fixed to a value of 1 kJ mol^{-1}; when left as a variable, it iterated to negative values.

The rotational dynamics of the complexes has been evaluated by using different rotational correlation times for the motion of the Gd–water oxygen and Gd–water proton vectors (τ_{RH} and τ_{RO}), as observed by ^{17}O NMR and ^{1}H NMRD.[26] Based on geometrical considerations, the ratio of those two rotational correlation times ($\tau_{RH}^{298}/\tau_{RO}^{298}$) has to lie between 0.65 and 1. The best least-square fits were obtained using parameters listed in Table 1. It is evident that the two monomeric compounds [Gd(DTTAP-bz-NO_2)$(H_2O)]^{2-}$ and [Gd(DTTAP-bz-NH_2)$(H_2O)]^{2-}$ are very similar and that the reduction of the nitro to an amino group does not significantly affect the relaxation properties.

In comparison to [Gd(DTPA)$(H_2O)]^{2-}$, the water exchange rates of [Gd(DTTAP-bz-NO_2)$(H_2O)]^{2-}$ and [Gd(DTTAP-bz-NH_2)$(H_2O)]^{2-}$ are significantly faster, which can be explained by the presence of the more bulky phosphinate. The space around the Gd(III) is more sterically crowded which forces the coordinated water molecule to leave. However, the faster water exchange has not much consequence on the r_1 value of the monomers as k_{ex}^{298} does not limit relaxivity for these small complexes. The relaxivities are higher than for current contrast agents due to the second sphere effect. Nevertheless, faster water exchange will be retained after conjugation of the monomer to a macromolecule, where the rotation is slowed down and the exchange rate becomes more

Table 1 Parameters resulting from simultaneous fits of ^{1}H NMRD and ^{17}O NMR data; r_1 values correspond to 37 °C and 0.47 T

Parameter	$[Gd(DTTAP\text{-}bz\text{-}NH_2)(H_2O)]^{2-}$	$[Gd(DTTAP\text{-}bz\text{-}NO_2)(H_2O)]^{2-}$	$[Gd(DTTAP\text{-}ph)(H_2O)]^{2-}$ [a]	$[Gd(DTPA)(H_2O)]^{2-}$ [b]
r_1/mM^{-1} s^{-1}	5.02	5.24	5.36	4.02
k_{ex}^{298} [10^6]/s^{-1}	8.9 ± 1.3	8.3 ± 0.6	11	3.3
$\Delta H^{\ddagger}$/kJ mol^{-1}	48.1 ± 3	48.5 ± 2	37.0	51.6
$\Delta S^{\ddagger}$/J mol^{-1} K^{-1}	+49.8 ± 10	+50.3 ± 8	—	+53.0
τ_{RO}^{298}/ps	116 ± 2	117 ± 10	296	58
E_R/kJ mol^{-1}	20.5 ± 0.8	18 ± 1	19	17.3
τ_V^{298}/ps	28.2 ± 0.5	26.3 ± 0.4	26	25
Δ^2 [10^{20}]/s^{-2}	0.403 ± 0.003	0.40 ± 0.01	0.32	0.46
D_{GdH}^{298} [10^{-10}]/m^{2}s^{-1}	25 ± 2	25 ± 2	22.75	20
E_{DGdH}/kJ mol^{-1}	30 ± 1	30 ± 1	—	19.4
δg_L^2 [10^{-2}]	3.3 ± 0.6	2.8 ± 0.5	8	1.2
$\tau_{RH}^{298}/\tau_{RO}^{298}$	0.81 ± 0.04	0.85 ± 0.08	0.37	—

[a] Ref. 2. [b] Ref. 21.

critical. The other parameters obtained in the fit have values comparable to analogous small paramagnetic Gd(III) complexes.

Fig. 3 displays the experimental and fitted ^{1}H NMRD profiles at 37 °C, as well as the calculated contribution from the second and outer sphere water. It is shown that the major contribution to the overall relaxivity comes from the inner sphere water and a very significant contribution is represented by the outer sphere. The second sphere contribution makes up to about 20% of the total relaxivity.

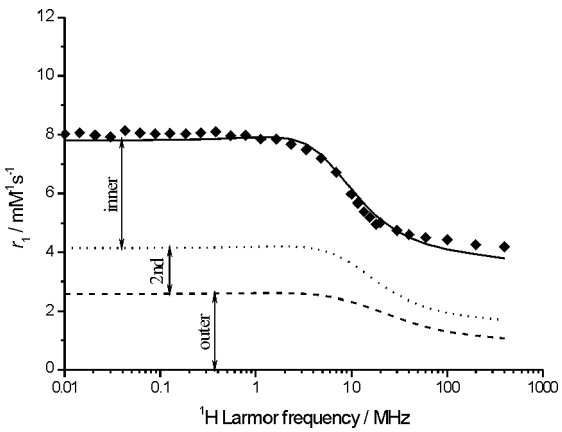


Fig. 3 ^{1}H NMRD profile of $[Gd(DTTAP\text{-}bz\text{-}NH_2)(H_2O)]^{2-}$ recorded at 37 °C (◆) with the fitted curve (full line) and calculated contributions form outer (dashed line) and second (dotted line) spheres.

^{17}O NMR and ^{1}H relaxivity measurements on the dendrimer complex G5-(Gd(DTTAP))$_{63}$

The ^{17}O NMR measurements were referenced to an Y(III) complex solution of the same pH and concentration as the Gd(III) analogue. The reason for using the diamagnetic Y(III) complex was a difference (at low temperatures) between T_1 values of the acidified water reference and the Y(III) complex. Similarly to previous observations on dendrimeric systems[20] T_1 values of the Y(III) complex were up to 30% lower than those of the acidified water. The measurements were performed at two magnetic fields, 9.4 T (54.2 MHz) and 4.7 T (27.1 MHz), and it was shown that ^{17}O T_1 strongly depends on the magnetic field. Fig. 1C shows the reduced longitudinal and transverse relaxation rates as a function of temperature.

Proton relaxivities of the dendrimer G5-(Gd(DTTAP))$_{63}$ were measured as a function of the ^{1}H Larmor frequency at temperatures 5, 15, 25, 37 and 50 °C and pH 6.25. The shape of the NMRD curves of the dendrimer complex shows a hump at higher frequencies, typical for macromolecules, with a maximum at ~30 MHz. In comparison to the monomers, a great relaxivity increase is observed (at 0.47 T and 37 °C the relaxivity is 26.8 mM^{-1}s^{-1} for G5-(Gd(DTTAP))$_{63}$ *vs.* 5.02 and 5.24 mM^{-1}s^{-1} for $[Gd(DTTAP\text{-}bz\text{-}NH_2)(H_2O)]^{2-}$ and $[Gd(DTTAP\text{-}bz\text{-}NO_2)(H_2O)]^{2-}$, respectively).

This relaxivity hump results from the slow rotation of the dendrimer. The relaxivity measured at the maximum of the hump (30 MHz) increases with temperature up to ~25 °C and decreases then if the temperature is increased further (Fig. 4). This implies that at lower temperatures the water exchange rate is a limiting factor for r_1, while at higher temperatures the relaxivity is mainly limited by rotation which is too fast. This trend is in contrast to that reported previously for dendrimeric conjugates. For the fast exchanging EPTPA derivatives, the relaxivities increase with decreasing temperature in the whole temperature range, showing that rotation limits r_1,[20] while for other dendrimeric complexes based on DOTA or DTPA an opposite trend was reported, as a result of slow water exchange.[11]

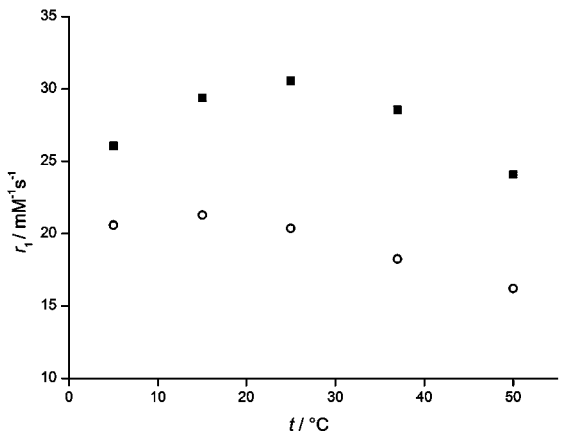


Fig. 4 Temperature dependence of the relaxivity of G5-(Gd(DTTAP))$_{63}$. The experimental data correspond to 30 MHz (■) and 1 MHz (○) of ^{1}H Larmor frequency.

The longitudinal ^{17}O relaxation rates were analysed by using the Lipari–Szabo approach[16,17] that distinguishes between rotational correlation times for the fast local and slow global motion of the complex. The local rotational correlation time is related to the motion of the Gd–water oxygen vector while the global rotational correlation time describes the slow overall motion of the entire macromolecule that the model considers spherical.[1,9] The degree of spatial restriction of the local motion with regard to the global rotation is given by the model free generalized order parameter S^2. For a completely free internal motion $S^2 = 0$, while for an internal motion completely correlated to the global one $S^2 = 1$. The second sphere contribution has been also included in the model, as described above for the monomers. The equations used for the calculations are listed in the ESI†.

All Gd–O and Gd–H distances were fixed at the same values as for the monomers as well as the hyperfine and quadrupolar coupling constants. Finally, we were not able to fit simultaneously ^1H NMRD and ^{17}O NMR data. The observed temperature dependence of the relaxivities in the high-field 'hump' could only be reproduced by a dramatically lower $\Delta H^{\ddagger}$ as compared to the value calculated from ^{17}O NMR. Therefore only the ^{17}O NMR data were fitted. The transverse ^{17}O relaxation rates allow an accurate determination of the water exchange rate, while the longitudinal ^{17}O relaxation rates give access to the rotational parameters.

The parameters obtained are shown and compared to an analogous dendrimer, G5-(Gd(EPTPA))$_{111}$[20] in Table 2. The local correlation time (τ_l) is much shorter than the global one (τ_g), 71 ps *vs.* 4417 ps, and the general order parameter S^2 is relatively small (0.28) which indicates an important flexibility of the Gd(III) chelates. The motion of the Gd(III) segments on the dendrimeric surface is less limited by spatial restrictions than in the similar PAMAM G5 chelate G5-(Gd(EPTPA))$_{111}$[20] or in Gadomer 17[12] and does not fully take advantage of the slow global motion of the molecule.

The water exchange rate slightly decreased upon conjugation to the dendrimer (5.0×10^6 s^{-1}, Fig. 1C). A similar trend was previously observed on conjugation to inulin[15] and on a smaller dendrimeric complex G2-(Gd(DO3A-P^{ABn}))$_{16}$.[3] This can be due to the possible electrostatic interactions between primary amines of dendrimer and negatively charged chelate, which changes the electron density of the chelate and therefore also the interaction with the inner sphere water. All parameters obtained can be found in Table 2 or in the ESI†.

Table 2 Selected parameters obtained from the ^{17}O NMR fit of G5-(Gd(DTTAP))$_{63}$] compared to an analogous dendrimer;[20] r_1 values correspond to 37 °C and 0.47 T

Parameter	G5-(Gd(DTTAP))$_{63}$	G5-(Gd(EPTPA))$_{111}$
r_1^{310}/mM^{-1}s^{-1}	26.8	23.9
k_{ex}^{298} [10^6]/s^{-1}	5.0 ± 0.2	150 ± 30
$\Delta H^{\ddagger}$/kJ mol^{-1}	56.2 ± 1	20.0 ± 4
$\Delta S^{\ddagger}$/J mol^{-1}K^{-1}	+71.9 ± 3	−21 ± 12
τ_{gO}^{298}/ps	4417 ± 340	4040 ± 300
E_{gO}/kJ mol^{-1}	21 ± 2	25 ± 2
τ_{lO}^{298}/ps	71 ± 3	150 ± 15
E_{lO}/kJ mol^{-1}	21 ± 2	31 ± 3
S^2	0.28 ± 0.01	0.43 ± 0.03
δg_L^2 [10^{-2}]	1.7 ± 0.1	—

Comparison with other macromolecular Gd(III) chelates

The overall relaxivity of the dendrimer G5-(Gd(DTTAP))$_{63}$ at 37 °C and 0.47 T is 26.8 mM^{-1} s^{-1}, higher than that of the inulin-conjugate.[15] This is directly proportional to the increase in the molecular weight (see Table 3). As a consequence of the second sphere water, the relaxivity of G5-(Gd(DTTAP))$_{63}$ is even higher than that of the similar dendrimeric conjugate[20] G5-(Gd(EPTPA))$_{111}$. Table 3 compares the principal parameters of several monomeric and macromolecular complexes, all of them derived from DTPA[21] or DOTA (Gadomer 17,[12] G2-(Gd(DO3A-P^{ABn}))$_{16}$[3]). The rigidity is described by the parameter S^2 and one can see that the internal flexibility increases from Gadomer 17 towards PAMAM G5 dendrimers. The very low local correlation time compared to Gadomer 17 is due to less restricted local motion.[20] ^1H NMRD profiles at 37 °C of various Gd(III) complexes are compared in Fig. 5.

Conclusion

A DTPA-based ligand bearing one phosphinic acid was conjugated to a generation 5 polyamidoamine (PAMAM) dendrimer *via* a benzylthiourea linkage. Gd(III) complexes of the monomers [Gd(DTTAP-bz-NO$_2$)(H$_2$O)]$^{2-}$ and [Gd(DTTAP-bz-NH$_2$)(H$_2$O)]$^{2-}$ and the dendrimeric G5-(Gd(DTTAP))$_{63}$ were synthesised and characterised by ^1H NMRD and ^{17}O NMR to evaluate parameters relevant to potential MRI applications. The experimental data were analysed by using the Solomon–Bloembergen–Morgan set of equations, extended by the Lipari–Szabo approach for the dendrimer. A contribution from the second

Table 3 Comparison of principal parameters for various Gd(III) complexes; r_1 values correspond to 37 °C and 0.47 T

Complex	τ_M^{298}/ns	k_{ex}^{298} [10^6]/s^{-1}	τ_{gO}^{298}/ps	τ_{lO}^{298}/ps	S^2	r_1/mM^{-1}s^{-1}	M_w/kDa	Ref.
[Gd(DTPA)(H$_2$O)]$^{2-}$	303	3.3	—	58	—	3.7	0.57	21
[Gd(DTTAP-bz-NH$_2$)(H$_2$O)]$^{2-}$	113	8.9	—	116	—	5.02	0.69	This work
inulin-(Gd(DTTAP))$_{23}$	170	5.9	—	866	—	18.3	23	15
G2-(Gd(DO3A-P^{ABn}))$_{16}$	30	33	—	—	—	14.1[a]	11.8	3
G5-(Gd(DTTAP))$_{63}$	199	5.0	4417	71	0.28	26.8	64	This work
G5-(Gd(EPTPA))$_{111}$	6.7	150	4040	150	0.43	23.9	110	20
Gadomer 17	100	1	3050	760	0.50	17.18	17.5	12

[a] J. Rudovský *et al.*, unpublished results.

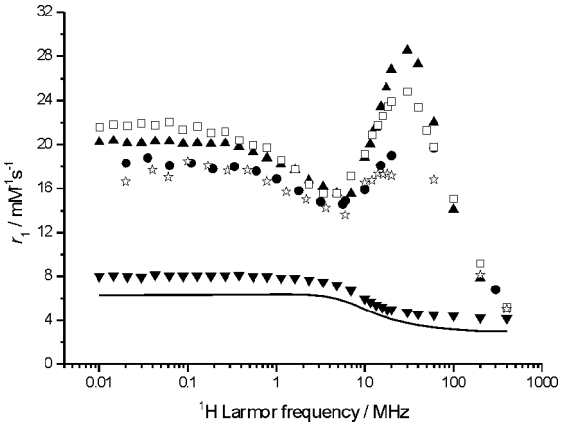


Fig. 5 Comparison of ^{1}H NMRD profiles at 37 °C of Gd(III) complexes: $[Gd(DTPA)(H_2O)]^{2-}$ (line), $[Gd(DTTAP\text{-}bz\text{-}NH_2)(H_2O)]^{2-}$ (▼), Gadomer 17 (⋆), inulin-$(Gd(DTTAP))_{23}$ (●), G5-$(Gd(EPTPA))_{111}$ (□) and G5-$(Gd(DTTAP))_{63}$ (▲).

hydration sphere was included in the analysis of the longitudinal relaxation rates.

The rigidity of the dendrimer complex, expressed by the term S^2, is lower than for the analogous G5-$(Gd(EPTPA))_{111}$,[20] probably due to the limited loading of G5-$(Gd(DTTAP))_{63}$. Despite this lower rigidity, G5-$(Gd(DTTAP))_{63}$ has a high relaxivity at higher magnetic fields, related to the presence of second sphere water molecules.

The unsubstituted amino groups on the dendrimer surface may offer a convenient way to further derivatization of the macromolecule. The attachment of targeting groups could allow for delivering the contrast agent to a place of interest in the body. Functionalization of the dendrimer with other probes can lead to multimodal imaging agents (dyes for near infrared imaging, radioactive tracers for SPECT, *etc.*).

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