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Poly(amidoamine) Dendrimer Based MRI Contrast Agents Exhibiting Enhanced Relaxivities Derived via Metal Pre-ligation Techniques

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This report presents the preparation and characterization of three [Gd-C-DOTA]⁻¹-dendrimer assemblies by way of analysis, NMRD spectroscopy and Photon Correlation Spectroscopy (PCS). The metal-ligand chelates were preformed in alcohol media prior to conjugation to generation 4, 5 and 6 PAMAM dendrimers. The dendrimer-based agents were purified by Sephadex[®] G-25 column chromatography. The combustion analysis, SE-HPLC and UV-Vis data indicated chelate to dendrimer ratios of 28:1, 61:1 and 115:1 respectively. Molar relaxivity measured at pH 7.4, 22°C, and 3T (29.6, 49.8 and 89.1 mM⁻¹s⁻¹) indicated the viability of conjugates as MRI contrast agents. 1/T1 NMRD profiles were measured at 23°C and indicated that at 22 MHz the 1/T1 reached a plateau at 60, 85 and 140 mM⁻¹s⁻¹ for the generation 4, 5 and 6 dendrimer conjugates, respectively. The PCS data showed the respective size of 5.2, 6.5 and 7.8 nm for G-4, 5, and 6 conjugates.

During the development of MRI, contrast agents have been employed to induce additional contrast and increase the sensitivity of MRI scans.(1,2) Increased usage of MRI, combined with the necessity for a contrast agent, prompts development of new efficient agents. Clinically used low molecular weight extracellular contrast agents, *e.g.* [Gd(DTPA)(H₂O)]⁻² (Magnevist[®]) suffer from rapid extravasation from blood vessels into interstitial space and fast decrease in concentration in blood vessels, combined with rapid whole body clearance.

In recent years, dendrimers have become an exciting class of polymeric platforms for assembly of multifunctional macromolecular nanomaterials undergoing pre-clinical development as diagnostics and therapeutic delivery vehicles. There have been reports indicating that dendrimers are applicable as carriers for site-specific delivery of drugs and that they do not alter the function of the molecules attached.(3–8) It has also been discovered that dendrimers can act as drugs themselves.(9,10) In the MRI field, dendrimers have not only allowed molecules to retain their function, but have also greatly enhanced the signal to noise ratio of various imaging modalities; however, this application has never reached its full potential.

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Supporting Information Available

Detailed description of syntheses, molar relaxivity measurements, relaxometry, photon correlation spectroscopy (PCS) and zeta (ζ) potential, UV-Vis Spectroscopy and figures. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

The use of Gd(III) chelates conjugated to high molecular weight carriers such as a PAMAM dendrimer prolongs intravascular retention and circulation time, slows down molecular rotation which results in a short relaxation time, and increases in relaxivity.(11–13) Polyamidoamine (PAMAM) dendrimers of different generations conjugated to acyclic diethylenetriamine pentaacetic acid (DTPA) derivatives, *e.g.*, 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid (1B4M-DTPA), are versatile in MRI applications due to their mono-dispersed nature and available range of molecular sizes.(14,15)

An acceptable contrast agent must be non-cytotoxic, must have high water solubility most preferably at physiological pH, and high relaxivity properties. The recent reports regarding nephrogenic systemic fibrosis (NSF) linked to the use of $[\text{Gd DTPA}]^{-2}$ derived MR contrast agents provided our laboratory with an impetus to re-evaluate use of bifunctional DTPA agents for creation of macromolecular MR agents not only in regards to complex stability, (16–20) but also in regards to their inherent extended *in vivo* residence time. $[\text{Gd(DOTA)}]^{-1}$ (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid) is known to be a better contrast agent in terms of complex stability and thus potentially less toxicity as compared to $[\text{Gd(DTPA)}]^{-2}$.(21,22) $[\text{Gd(DOTA)}]^{-1}$, DOTAREM™, has been extensively evaluated in patients with pre-existing renal diseases and to date without clinical side effects.(23) The higher relaxivity and kinetic stability of $[\text{Gd(DOTA)}]^{-1}$ makes it an alternative contrast agent for MRI.(24) The formation and stability of the $[\text{Gd(DOTA)}]^{-1}$ complex has also been thoroughly studied; dissociation of the metal ion was exceedingly slow even at low pH (2–4), on the order of days,(25) while its thermodynamic stability was comparable to $[\text{Gd(III)}]$ (DTPA).(26) $[\text{Gd(DOTA)}]^{-1}$ was previously shown to be five orders of magnitude higher in *in vitro* stability as compared to $[\text{Gd(DTPA)}]^{-2}$, which potentially translates to decreased *in vivo* toxicity.(21)

Herein, we report the preparation and characterization of three contrast agents with different generation (4, 5 and 6) of dendrimers. The C-DOTA (2-(4-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid) ligand is first used to sequester Gd(III) (Scheme 1) and thereafter the formed metal complex is covalently attached to the terminal –NH₂ groups of the dendrimer. We have recently reported that pre-metallation method to be significantly advantageous over conventional post-metallation methods, resulting in both a more directly characterizable product possessing an ~2-fold enhancement of molar relaxivity when using a bifunctional DTPA chelating agent while also decreasing the overall Gd(III) content by ~30%.(27) We have also reported the applicability of a C-DOTA derivative applied to complex Gd(III) as an agent of choice to replace the use of bifunctional DTPA agents employed to sequester Gd(III) as a component of macromolecular MR contrast agents.(28)

Table 1 summarizes the number of chelates per dendrimer obtained from analysis and SE-HPLC. For comparison, a UV-Vis spectrophotometric titration method was developed. A standard curve using compound **2** (Figure 1; Supporting Information), which represents a surrogate of the chelate conjugated to an amino surface on the dendrimer, was generated from this method and is shown in Figure 2 (Supporting Information). The results from assessing the number of chelates per dendrimer were collected in Table 1. Clearly, there is agreement between the two methods within experimental error indicating that spectrophotometric method also to be a viable method for the determination of the number of chelates conjugated to a dendrimer platform.

A plot of the inverse of longitudinal relaxation time ($1/T_1$) versus Gd(III) concentration for the three dendrimer conjugates and Magnevist™ is shown in Figure 3, while the relaxivity values obtained as a slope of this plot are summarized in Table 1. The data showed that the relaxivity of the three dendrimer based agents are 7–21 times higher than that of

Magnevist™. This indicates that G6-(C-DOTA-Gd)₁₁₅ might be applicable in the submicromolar range and thus also presents the highest potential to eliminate concerns regarding Gd(III) toxicity.

The relaxivity values (30, 50 and 90 mM⁻¹s⁻¹) reported here produced by macromolecules measured at 3T field strength might seem high, but the reality is that these numbers are not uncommon. A relaxivity range of 139–237 mM⁻¹s⁻¹ at 2.4 T field strength, which is close to our field strength, has been reported by Zhang et al in their contemporary work with fullerene.(29) The solutions prepared were based on the gadolinium content from the ICP report of the purified dendrimer conjugate. We reported the efficiency of this macromolecular purification method earlier,(27) which is also utilized by other researchers.(29)

In general, conjugation of compound **1** [Gd(C-DOTA)]⁻¹ (Scheme 1) to a higher generation dendrimer such as G6 would result in a more hydrophobic conjugate due to the replacement of exterior primary amines with the complexes. Hydrophobic interaction with solvent molecules is known to slow down the tumbling time (τ_R) which results in increase of the relaxivity.(30–32) Based on the combustion analysis, SE-HPLC and UV-Vis data, the agent based on the G6 has the highest free amino sites (141), and thus it is expected to have the highest relaxivity. The experimental data (Table 1) support this position.

Consideration of the nuclear magnetic relaxation dispersion (NMRD) for a given contrast agent is essential since the field strength of clinical MRI instrumentation ranges from 2 to 180 MHz. The 1/T₁ NMRD profiles of the G4, G5 and G6 dendrimer conjugates at 23°C are depicted in Figure 4, wherein the relaxivity for all three agents peaked at ~22 MHz. This behavior is similar to a previously reported measurement using G5, G7, G9 and G10 dendrimers in which the relaxivities peaked at ~22 MHz.(33,34) Henry et. al. pointed out that the relaxivity of G7 conjugate is comparable to that of G6, which means G6 is the highest generation dendrimers where a significant increase in relaxivity can be observed based on the simple size and amine number of the dendrimers platform.(33,34) However, whether that result will hold true for the agents prepared employing the pre-metallation method presented here remains to be determined in future studies.

The size of conjugates determined here is based on 1 μM stock solution prepared in ambient water at pH = 6 to eliminate buffer interference (Table 2). As noted earlier, size measurement based on PCS is concentration dependent (35) and also buffer dependent.(36) As expected, the G6 conjugate has the largest size (Table 2) and thus has the highest impact on the relaxivity (Table 1). This is the first time that our laboratory has measured the size of PAMAM dendrimer conjugated to [Gd(C-DOTA)]⁻¹. Relaxivity was observed to be linearly correlated to the size of chelated dendrimer conjugate; the larger the size, the greater the relaxivity (Figure 5; Supporting Information). This is in agreement with a previous report utilizing a similar [Gd(DO3A)]-derivative by Margerum *et. al.*(36), and other authors where subsequent increases in size and molecular weight results in increased relaxivity.(33,34) This is also a major benefit from attaching a small chelate to a large macromolecule in which the associated tumbling time of the molecule increases leading to increases in relaxation enhancement.(37) Our NMRD data showed that the relaxivity of G=6 dendrimer chelate is 140 mM⁻¹s⁻¹ at 22 MHz, which is slightly lower than Gd(III)-glutamine synthetase (148 mM⁻¹s⁻¹) at 22.5MHz.(38,39)

The measurement of zeta potential (ζ) reveals the accumulation of ions at the particle surface.(35) The overall surface charge or ζ potential of the G6 agent is the highest due to the highest density of surface charge as expected. Also, high positive charge ζ potential is indicative of high electrostatic repulsion between particles and provides an energy barrier

against aggregation.(35) Since the differences in ζ potential of the agents is not significant based on the data obtained, one can assume a likelihood of each agent to aggregate to be low. This will be advantageous for *in vivo* applications where aggregation could interfere with glomerular filtration clearance through kidney, which would be the preferred route,(40) especially for larger G5 and G6 conjugates.

In summary, this study indicates that macromolecular-based MRI contrast agents prepared here are efficient and effective in modulating and relaxing water protons when compared to a single Gd(III) chelate. Our study also shows that appending the pre-formed Gd(III) complex to the dendrimer is far more convenient and significantly more advantageous in areas including species distribution, ease of characterization, stability, and solubility. As reported earlier, this pre-metallation strategy also produces a more hydrophobic agent that results in high relaxivity, and faster blood clearance rate *in vivo*.(27) The characterized data provide us with valuable information regarding the nature of the conjugates such as the size and the surface charge, and also enhance our understanding of how these parameters impact the relaxivity and to a certain degree what we can expect from these agents *in vivo*. These data strongly suggest that the G6-(C-DOTA-Gd)₁₁₅ is a superior agent based on its high relaxivity which will also enable use of a much lower injected dose for all future studies thereby facilitating also translation into clinical trial evaluation.

Experimental

See supporting information

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 3. Molar relaxivity plots of G4-(C-DOTA-Gd)₂₈ (■; 29.6 mM⁻¹s⁻¹), G5-(C-DOTA-Gd)₆₁ (●; 49.8 mM⁻¹s⁻¹), G6-(C-DOTA-Gd)₁₁₅ (▲; 89.1 mM⁻¹s⁻¹) and Magnevist (◆; 4.2 mM⁻¹s⁻¹).

Figure 4. $1/T_1$ NMRD profiles of 1 mM solution of prohance (▲), Magnevist® (●), G4-(C-DOTA-Gd)₂₈(-), G5-(C-DOTA-Gd)₆₁(◆), G6-(C-DOTA-Gd)₁₁₅(■), as a function of frequencies at 23°C.

Scheme 1.
Synthesis of dendrimer conjugates.

Table 1

Number of chelates and molar relaxivity values measured for G4-(C-DOTA-Gd)₂₈, G5-(C-DOTA-Gd)₆₁ and G6-(C-DOTA-Gd)₁₁₅.

Agent	No. chelate (analysis)	No. chelate (UV)	r_1 (mM ⁻¹ s ⁻¹)
Magnevist	---	---	4.2
G4-(C-DOTA-Gd) ₂₈	28	26	29.6
G5-(C-DOTA-Gd) ₆₁	61	58	49.8
G6-(C-DOTA-Gd) ₁₁₅	113	107	89.1

Table 2Values of size and ζ potential for free and conjugated dendrimers.

Generation	Predicted Diameter* (nm)	Measured Diameter (nm)	ζ Potential
G4	4.0	4.2	0.1
G4 Conjugate		5.2	-60
G5	5.3	5.1	7.6
G5 Conjugate		6.5	-65
G6	6.7	6.4	17.4
G6 Conjugate		7.8	-69

* Predicted values by Tomalia. (41)