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Improving T_1 and T_2 magnetic resonance imaging (MRI) contrast agents through the conjugation of an esteramide dendrimer to high water coordination Gd(III) hydroxypyridinone (HOPO) complexes

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Abstract

Commercial gadolinium magnetic resonance imaging (MRI) contrast agents are limited by low relaxivity (r_1) and coordination to only a single water molecule ($q = 1$). Consequently, gram quantities of these agents must be injected to obtain sufficient diagnostic contrast. In this study, MRI contrast agents for T_1 and T_2 relaxivity were synthesized using hydroxypyridinone (HOPO) and terephthalamide (TAM) chelators with mesityl and 1,4,7-triazacyclononane (TACN) capping moieties. When covalently conjugated to a highly biocompatible esteramide dendrimer, T_2 relaxation rates up to $52 \text{ mM}^{-1}\text{s}^{-1}$ and T_1 relaxation rates up to $31 \text{ mM}^{-1}\text{s}^{-1}$ per gadolinium are observed under clinically relevant conditions. These values are believed to be brought about by using a dendritic macromolecule to decrease the molecular tumbling time of the small molecule complexes. These agents also show high aqueous solubility and low toxicity *in vitro*. In this study we report six new compounds: three discrete complexes and three dendrimer conjugates.

Keywords

MRI contrast agent; hydroxypyridinone (HOPO); relaxivity; 1,4,7-triazacyclononane (TACN); terephthalamide (TAM); dendrimer; esteramide (EA) dendrimer; drug delivery; gadolinium

Introduction

Magnetic resonance imaging (MRI) utilizes paramagnetic Gd(III) contrast agents (CA's) to distinguish background tissue from regions of diagnostic interest. MRI CA's provide clarity in images of cancer, atherosclerosis, stroke, and other abnormalities, revealing their exact location and size. Currently, ten Gd(III) CA's are approved for clinical use worldwide: Gd-DTPA (Gd-diethylene triamine pentaacetic acid, Gadopentetate), Gd-DOTA (Gd-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, Gadoterate) and their derivatives. These agents have suboptimal water exchange rates (i.e. slower than the Larmor frequency of clinical MRI imagers ideal for this class of agent) and bind only a single water molecule in the inner metal coordination sphere ($q = 1$). These parameters contribute to low relaxivity (r_1 is a function of T_1 relaxation time and r_2 is a function of T_2 relaxation time; measured in $\text{mM}^{-1}\text{s}^{-1}$), a determinant of the length of water or proton exchange times. For Gd-DOTA, $r_1 = 3.0 \text{ mM}^{-1}\text{s}^{-1}$ and $r_2 = 3.9 \text{ mM}^{-1}\text{s}^{-1}$, therefore, gram quantities of these agents must be administered in order to obtain an adequate image contrast (1-4).

According to the Solomon-Bloembergen-Morgan (SBM) Equations (5-7), relaxivity can be increased by having shorter water exchange times (τ_m), a slower tumbling rate ($1/\tau_r$), and a greater number of inner-sphere water molecules (q). Current MRI contrast agent prototypes improve on all three of these parameters as compared to Gd-DTPA and Gd-DOTA families of clinically approved commercial agents (8-9).

Previous research in the Raymond group has developed MRI contrast agents for T_1 applications with fast water exchange (τ_m) and higher q values (10-11). In 2007, the first $q = 3$ Gd(III) contrast agents with fast water exchange and high thermodynamic stability were developed, though the use of the 1,4,7-Triazacyclononane (TACN) cap and hexadentate hydroxypyridinone (HOPO) ligands (12, Figure 1).

Increasing relaxivity by slowing the molecular tumbling rate ($1/\tau_r$) has been achieved by conjugation to macromolecules. Increases in molecular weight by addition to a dendrimer (13-15) protein (16-18), nanoparticle (19), or nanodiamond (20) has effectively increased T_1 relaxivity in Gd-DOTA, Gd-DTPA, and Gd-HOPO ligand chelator systems. However, this increase in molecular weight will ideally be accompanied by additional mechanisms of excretion, as macromolecules larger than ~40 kDa are not easily cleared from the body. Dendrimers of this size developed in the Fréchet group have been shown to be highly biocompatible and effective macromolecules for drug delivery (21-24), with favorable bioaccumulation and excretion profiles. The structure of these dendrimers can be readily modulated to incorporate slow degradation *in vivo*, thus facilitating excretion as exemplified by the esteramide dendrimers used for this study (25, Figure 2). In addition to increasing the relaxivity of the gadolinium, the dendrimer can also provide high water solubility, protection from premature enzymatic degradation, and longer blood residence times, leading to an overall dramatic improvement in the pharmacokinetic profile of the agent.

Excretion profiles are of importance in gadolinium based CAs because a disorder known as Nephrogenic Systemic Fibrosis (NSF) has been associated with renal deficient patients receiving acyclic Gd(III) contrast agents. In these patients lacking a proper excretion mechanism due to severe kidney dysfunction, small molecule contrast agents not excreted within several days or weeks can dissociate gadolinium, leaving the toxic ligands and free Gd(III) ions in the body. This Gd(III) has a high affinity for Ca(II) ion channels and has been associated with a thickening of organs and skin (1). NSF is currently incurable, although preventative measures and efforts to couple therapy with dialysis therapy have been explored (26). Novel contrast agents with higher relaxivity per-Gd would require injection of a much lower dose, which coupled to stronger binding of the gadolinium by the ligand, would contribute to enhanced patient safety. Additionally, because water soluble macromolecules below 40 kDa tend to be excreted well from the body and do not passively cross cell membranes, covalent attachment of strongly binding chelates to dendrimers and similar macromolecules may allow for easier excretion of Gd(III) than that observed with free small molecule chelates.

Much attention is paid to T_1 relaxivity, or positive mode imaging, as this is the main mode of imaging currently in use. T_1 contrast is inherently easier to improve as tissue has a slower T_1 relaxation rate than T_2 under physiological conditions. However, contrast agents that are versatile for both T_1 and T_2 relaxivity would be potentially be useful for improving scans taken in either imaging mode. Gd(III) contrast agents with highly desirable attributes according to the SBM Equation also exhibit high T_2 relaxivity values, and some have acceptable r_2/r_1 values to enhance T_2 images as well. High T_2 relaxivity contrast agents are used for negative mode imaging, preferred for imaging softer tissue. Currently, clinical T_2 image enhancement is achieved through paramagnetic Fe(III) agents (27), however, a single

versatile agent such as current extracellular Gd(III) agents provide patients with fewer opportunities for adverse medication reactions and clinical issues.

Results/Discussion

Complex Synthesis & Characterization

Previously synthesized TACN-capped HOPO ligands lacked a synthetic handle to which a dendritic macromolecule could be attached (12). A novel TACN capped complex was synthesized with two (1-Me)-3,2-HOPO ligands and a functionalized terephthalamide (TAM) ligand, for a hexadentate all-oxygen donor Gd(III) complex (see Supporting Information for experimental detail). Gd-TACN-bis(1-Me)-3,2-HOPO-TAM-ethylamine was conjugated via an amide bond to the esteramide dendrimer, which can theoretically yield up to eight complexes per dendrimer (Scheme 1). In the case of TACN capped ligands such as **4**, actual loading was typically significantly lower than the theoretical limit. Since small molecule complex **4** was different than the tris-(1-Me)-3,2-HOPO ligands previously described (Werner et al. 2007), an analogue of this with an ethyl functionality, Gd-TACN-bis(1-Me)-3,2-HOPO-TAM-ethyl, **5**, was synthesized for relaxivity and cytotoxicity comparison to the novel complex and conjugated macromolecule (Figure 3). Table 1 shows the relaxivity measurements for these small-molecule complex and dendrimer conjugated complexes.

Previously synthesized mesityl-capped HOPO ligands had high q values of 2-3, but lacked the solubility ($<100 \mu\text{M}$ in water) required for clinical use. Gd-Mesbis(1-Me)-3,2-HOPO-TAM-ethylamine and Gd-Mes-bis(1-Me)-3,2-HOPO-TAM-N3 (Figure 4, 9) were conjugated to the esteramide dendrimer as above with the expectation that the PEGylated dendritic macromolecule would suitably solubilize them. Not only did this impart improved aqueous solubility to the complexes, but also substantially increased the per gadolinium r_1 and r_2 relaxivities over those of the small molecules. (Table 1). Furthermore, while the TACN capped ligands above typically exhibited low (12-13%) loading on the dendrimer according to ICP, mesityl capped ligands **7** and **8** were attached to the dendrimer with an average of seven and six Gd(III) complexes per dendrimer, respectively (See Supporting Information).

Additionally, a mesityl complex with a PEG450 (**9**) was compared with a novel branched mesityl complex, Gd-Mes-bis(1-Me)-3,2-HOPO-TAM-Asp-Asp₂-12OH (Figure 5). With the TREN capped system, the branched dendrimer had a larger T_1 relaxivity and it was sought to compare both the T_1 and T_2 relaxivities of these higher q coordination compounds. Although solubility of these complexes was increased significantly ($> 1 \text{ mM}$) over their small molecule counterparts, the large doses required for MRI imaging would still exclude these complexes due to solubility.

T_1 & T_2 Relaxivity

Due to their coordination of two or three water molecules rather than one, the small molecule complexes show T_1 relaxivity values two to three times higher than that of commercial contrast agents ($7\text{-}9 \text{ mM}^{-1}\text{s}^{-1}$, Table 1). The T_2 relaxivities were relatively high for small molecules as well. The outer-sphere water exchange, which is a larger factor in T_2 than in T_1 (28-29), favors interaction with the TACN cap over the hydrophobic mesityl cap. The r_2/r_1 ratio (or T_2 relaxivity to T_1 relaxivity), is also important (Table 2). The small molecule contrast agents outperform the commercial contrast agents in this aspect as well.

The mesityl capped HOPO complexes show how relaxivity changes as the molecular weight and the shape of molecule are modified. The Gd-Mesityl-PEG450 complex has a similar molecular weight to that of the branched dendrimer Gd-Mes-Asp-Asp₂-12OH. However

despite their chelation and structural similarities, both their T_1 and T_2 relaxivities vary significantly differ from each other (Table 1). While the more dendritic branched mesityl (Asp-Asp₂-12OH) had a larger T_1 relaxivity than the small molecule, the mesityl with a PEG450 chain obtained a much larger increase in its T_2 relaxivity and therefore exhibits a much larger r_2/r_1 relaxivity ratio (Table 2). This was attributed to the much larger surface area of the PEG450 molecule as compared to the ASP-ASP₂-12OH molecule. The larger surface area allowed for many more outer sphere solvent interactions, significantly increasing the r_2 relaxivity. The branched dendrimer slows the tumbling of the gadolinium complex through its high molecular weight and steric restrictions, but coordinates fewer outer sphere water molecules due to its much lower hydrodynamic volume, leading to a larger increase in T_1 relaxivity than T_2 .

Both the Gd-TACN capped complex and the Gd-Mesityl capped complexes were conjugated to the esteramide dendrimer (taScheme 1). The esteramide dendrimer has eight complex loading sites in its core which are sterically hindered enough to slow the local motion of the Gd(III) along with slowing the rotational correlation time. This hindrance of molecular motion may explain why the T_1 and T_2 relaxivities per Gd(III) are substantially increased by this conjugation (Table 1).

Gd-TACN-bis(1-Me)-3,2-HOPO-TAM-ethylamine esteramide conjugate has a per Gd(III) relaxivity of 31 ($\text{mM}^{-1}\text{s}^{-1}$) for r_1 and 52 ($\text{mM}^{-1}\text{s}^{-1}$) for r_2 for a r_2/r_1 of 1.68. The T_1 relaxivity is about ten times that of the commercial contrast agents at physiological conditions (37 °C) and clinically relevant field strength (60 MHz). Although the relaxivity is much larger, it is done at the expense of increasing molecular weight by 40-50 fold. This is a limitation of this conjugate, as about a fivefold higher mass dose would be required clinically. The relaxivity values of the Gdmesityl complexes increased upon conjugation to the dendrimer, however, not as dramatically. However, upon conjugation to the esteramide dendrimer, these complexes, as well as those described above, obtained high solubility (> 50 mM) in water and exhibited no or minimal aggregation as measured by Dynamic Light Scattering (DLS, see Supporting Information). This is notable data for other potential MRI contrast agents, which exhibit high relaxivity through high q values and fast water exchange, but lack the required solubility under physiological conditions.

Cytotoxicity

Cytotoxicity of these compounds was conducted so that animal studies may be pursued in the future. Since the dendrimer has a murine *in vivo* excretion time of 48-72 h (25), Gd(III) complex-dendrimer conjugates must be evaluated over longer durations than small molecule cytotoxicity testing. Complexes and dendritic macromolecule conjugates exhibit no cytotoxicity (see Supporting Information) up to 25 μM over 72 hours using HeLa cells.

Conclusions

The relaxivity, solubility, and biocompatibility of several MRI contrast agents have been significantly enhanced in this study by covalent attachment to polymeric and dendrimer carriers, and novel small molecule T_1 and T_2 MRI contrast agents with fast water exchange and high q values have been synthesized and evaluated for biological suitability. Small molecules attached to either branched or linear dendritic macromolecules have been evaluated to show T_1 and T_2 relaxivity, solubility and suitability increases. An esteramide dendrimer was conjugated via peptide bond to $q = 2-3$ MRI contrast agents to slow molecular tumbling time and significantly increase T_1 and T_2 relaxivity under physiologically relevant conditions. Altering systemic parameters increased relaxivity and biocompatibility significantly in $q = 2-3$ systems. As a result of much higher per-Gd(III) relaxivity, less agent would be required, decreasing patient exposure to potentially toxic

Gd(III) ions. These agents also show substantially improved relaxivity and solubility over the small molecule complexes, making them attractive candidates for further exploration as next generation contrast agents.

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Figure 1. Previously developed hexadentate oxygen donor Gd(III)MRI contrast agents with fast water exchange and high q values (12). Left: Gd-TACN-*tris*-(1-Me)-3,2-HOPO (**1**). Right: Gd-TACN-*tris*-1,2-HOPO (**2**).

Figure 2.

Previously reported esteramide dendrimer (EA dendrimer, **3**) developed for drug delivery. The architecture is designed for stability for the duration of a complete MRI scan with biodegradability as an eventual excretion pathway (25).

Figure 3. Novel TACN capped complexes to increase T_1 and T_2 relaxivity. Gd-TACN-trisHOPO (12) has a $q = 3$ Gd-TACN-*bis*-(1-Me)-3,2-HOPO-TAM-ethylamine (left, **4**) was conjugated to the esteramide dendrimer and Gd-TACN-*bis*-(1-Me)-3,2-HOPO-TAM-ethyl (right, **5**) was used as a small molecule analogue to compare relaxivity and cytotoxicity.

Scheme 1.

Conjugation of the Gd-TACN-*bis*-(1-Me)-3,2-HOPO-TAM-ethylamine complex to the EA dendrimer (**6**). Conjugations to other ligands were carried out under similar conditions.

Figure 4.

Gd-Mes-bis(1-Me)-3,2-HOPO-TAM-ethylamine (left, **7**) and Gd-Mesbis(1-Me)-3,2-HOPO-TAM-ethylamine-bisethylamine (right, **8**) were originally synthesized to capitalize on high relaxivity stemming from a higher q value. However, these compounds were not suitable for MRI contrast agents due to their low solubility ($<10\ \mu\text{M}$ in water) and subsequent aggregation (9). Upon conjugation to the esteramide dendrimer, they become suitably soluble and have high relaxivities.

Figure 5. Mesityl capped ligands with branched dendrimer (Gd-Mes-*bis*-(1-Me)-3,2-HOPO-TAM-Asp-Asp₂-12OH, left, **9**) and PEGylated dendrimer (Gd-Mes-*bis*-(1-Me)-3,2-HOPO-TAM-PEG450, right, **10**). The branched dendrimer uses its rigidity to achieve a larger T_1 relaxivity and the PEGylated dendrimer uses its much larger surface area to achieve a larger T_2 relaxivity.

Table 1

Small molecule and macromolecular per Gd(III) r_1 and r_2 relaxivity data at 60 MHz and 37 °C.

Complex	r_1 complex ($\text{mM}^{-1}\text{s}^{-1}$)	r_1 Complex + Dendrimer ($\text{mM}^{-1}\text{s}^{-1}$)	r_2 Complex ($\text{mM}^{-1}\text{s}^{-1}$)	r_2 Complex + Dendrimer ($\text{mM}^{-1}\text{s}^{-1}$)
Mn-DPDP (Teslascan™) (27)	1.88 * at 20 mHz	--	2.18 * at 20 mHz	--
Gd-DTPA (Magnevists™) (1)	3.3 ± 0.3	--	3.9	--
Gd-DOTA (Dotarem™) (1)	3.0 ± 0.3	--	3.5	--
Gd-TACN-N1 (4)	9.89 ± 0.03	31.0 ± 0.4	16 ± 0.5	52 ± 2
Gd-Mes-N1 (7)	9.3 ± 0.2 *	19.6 ± 0.4	16.0 ± 0.2	29.2 ± 0.3
Gd-Mes-N3 (8)	7.2 ± 0.2 *	18.7 ± 0.3	12.6 ± 0.4	23.6 ± 0.3
Gd-TACN-ethyl (5)	7.27 ± 0.09	--	11.2 ± 0.2	--
Gd-Mes-Asp- Asp ₂ -12OH (9)	14.8 ± 0.4	--	17.4 ± 0.1	--
Gd-Mes-PEG450 (10)	12.01 ± 0.05	--	21.3 ± 0.3	--

Data listed novel to this study (Werner et al. 2009).

* Previously only reported at 298K and 20 MHz (11.9 for Mes-N1 and 9.9 Mes-N3).

Table 2

Relative ratios of r_2 relaxation rates compared with r_1 relaxation rates.

Complex	r_2/r_1
Mn-DPDP	1.16
Gd-DTPA	1.18
Gd-DOTA	1.17
Gd-TACN-N1	1.61
Gd-TACN-N1 + EA dendrimer	1.68
Gd-Mes-N1	1.72
Gd-Mes-N1 + EA Dendrimer	1.49
Gd-Mes-N3	1.75
Gd-Mes-N3 + EA Dendrimer	1.26
Gd-TACN-ethyl	1.54
Gd-Mes-Asp-Asp-12OH	1.18
Gd-Mes-PEG450	1.78