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Conjugation Effects of Various Linkers on Gd(III) MRI Contrast Agents with Dendrimers: Optimizing the Hydroxypyridinonate (HOPO) Ligands with Nontoxic, Degradable Esteramide (EA) Dendrimers for High Relaxivity

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Supporting Information

ABSTRACT: One essential requirement for more sensitive gadolinium-based MRI contrast agents is to slow the molecular tumbling of the gadolinium(III) ion, which increases the gadolinium's relaxivity (i.e., its ability to speed up the NMR relaxation of nearby water molecules). One route to this is through conjugation to high-molecular-weight polymers such as dendrimers. In this work, amine-functionalized TREN-bis(1,2-HOPO)-TAM-ethylamine and TREN-bis-(1-Me-3,2-HOPO)-TAM-ethylamine ligands have been synthesized and attached to biocompatible 40 kDa esteramide (**EA**)- and poly-L-lysine (**PLL**)-based dendrimers capable of binding up to eight gadolinium complexes. These conjugates have T_1 relaxivities of up to 38.14 ± 0.02 mM^{-1} s^{-1} per gadolinium at 37 °C, corresponding to relaxivities of up to 228 mM^{-1} s^{-1} per dendrimer molecule. This relaxivity expressed on a "per Gd" basis is several times that of the small-molecule complexes and an order of magnitude higher than that of current commercial agents. Because of their high performance and low toxicity, these macromolecules may constitute an attractive complement to currently available gadolinium(III)-based contrast agents.

In the last three decades, magnetic resonance imaging (MRI) has become one of the most prevalent medical-imaging modalities used in clinical radiology, with over 27.5 million MRIs performed in 2007. Paramagnetic gadolinium(III) contrast agents are used to enhance the signal in about one-third of these scans. This is done through the gadolinium's ability to speed up the relaxation of water molecules after they are disturbed by a magnetic field, a parameter known as relaxivity (r_1). Concern about gadolinium dosage and release has increased because of nephrogenic systemic fibrosis (NSF), an incurable thickening of tissue and skin seen in patients with late-stage renal failure. This condition appears to arise from the tendency of free gadolinium ions to bind to hydroxyapatite in bone tissue and transport it across cellular ion channels.[1] Increasing the chelate stability, relaxivity, and efficiency of contrast agents will allow for smaller doses and lower exposure to free gadolinium ions, improving their performance and lowering the risk for NSF by injection of smaller quantities of Gd(III).

All current commercial contrast agents use octadentate poly(amino)carboxylate ligands as scaffolds to coordinate gadolinium and contain only one inner-coordination water molecule ($q = 1$). Previous research in the Raymond laboratory has developed hexadentate oxygen donor chelators for gadolinium with stabilities similar to those of commercial poly(amino)carboxylate contrast agents by utilizing the oxophilicity of gadolinium. These tris(2-aminoethyl)amine (TREN)-capped ligands, which contain either two 1-Me-3,2-hydroxypyridinonate (HOPO) or two 1,2-HOPO rings and an amine-functionalized 2,3-dihydroxyterephthalamide (TAM) ring (Figure 1),[2−7] have at least two coordinated water molecules and exhibit high exchange rates, allowing a much higher theoretical relaxivity than the small-molecule amine-based chelators. These complexes exhibit high T_1 relaxivities (10−13 mM^{-1} s^{-1}) and thermodynamic complex stabilities (pGd ∼ 17−18). The 1-Me-3,2-HOPO-based ligands used in this study vary by the nature of the amine pendant from the TAM ring. Complex **1** has a short, rigid linker and a known q value of 2.[2] Complex **2** has a longer, more flexible linkage incorporating a second ethylamine moiety, and complex **3** has a branched linkage with a third ethylamine group. Complex **4**, which contains the 1,2-HOPO moiety, varies from these by its nitrogen substitution within the HOPO rings.

MRI contrast agents can be further optimized for higher relaxivity and solubility through conjugation to a biocompatible macromolecule such as a protein,[3] polypeptide,[8] dendrimer,[9−14] or nanoparticle,[15,16] which decreases the molecular tumbling time of the gadolinium as a result of the reduced number of degrees of freedom of large molecules in solution. We anticipated that the previously reported[17] esteramide (**EA**)- and branched poly-L-lysine (**PLL**)-based dendrimers would be promising, as these degradable dendrimers offer a synthetically straightforward route to high-molecular-weight conjugates with facile renal clearance and low toxicity. We also anticipated that the densely packed core of the dendrimers would sterically inhibit the motion of the gadolinium complex, further increasing its relaxivity. The **EA** and **PLL** dendrimers developed by the Fréchet group

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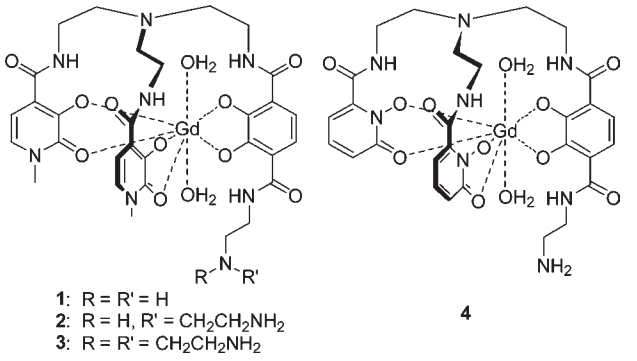


Figure 1. Gd−TREN-bis(1-Me-3,2-HOPO)-TAM-ethylamine complexes **1**−**3** and Gd−TREN-bis(1,2-HOPO)-TAM-ethylamine complex **4**.

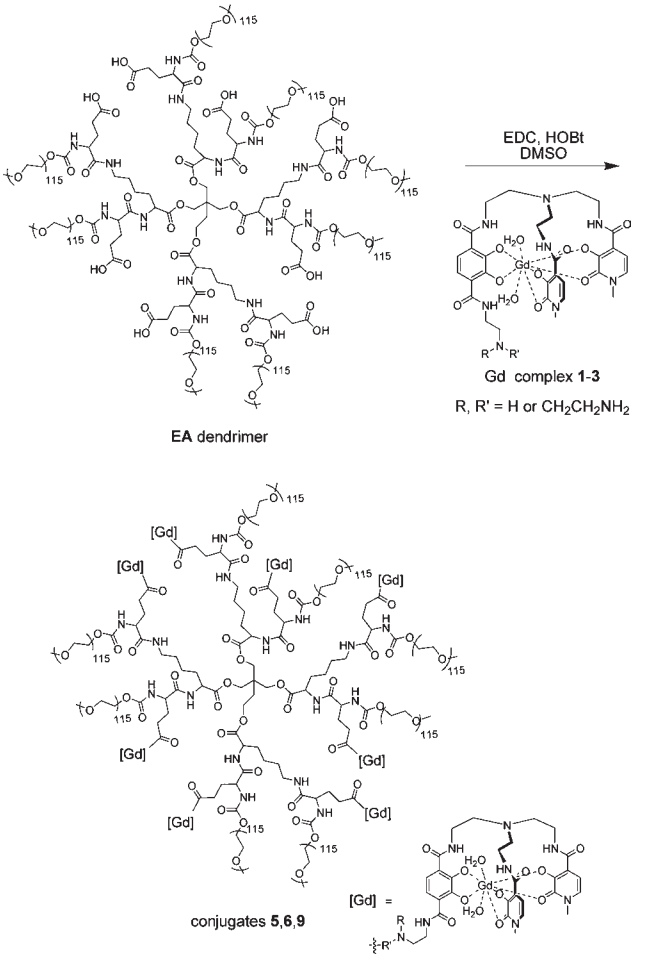
exhibit low toxicity in vivo and favorable degradation profiles (see the Supporting Information) and can be decorated with up to eight gadolinium complexes per dendrimer.[17,18] These dendrimers have also been shown to increase the half-life in blood serum and the residence time of small-molecule drugs, allowing for improved drug delivery in vivo.[19] In addition to enhancing the relaxivity and blood half-life of contrast agents, polymeric carriers are also expected to significantly reduce the toxicity of these agents because of the much lower rates of endocytosis for macromolecules in comparison with lipophilic small-molecule compounds such as non-polymeric gadolinium complexes. This should reduce the amount required for injection, thereby reducing the amount of gadolinium entering cells and facilitating its eventual excretion through the renal system.

We report herein that **EA** and **PLL** dendrimers have been successfully conjugated to precomplexed Gd−TREN-bis-HOPO-TAM-ethylamine based complexes. The conjugates described herein exhibit high relaxivities (r_1) of up to 38 mM^{-1} s^{-1} under the clinically relevant conditions of 37 °C at 60 MHz, which is almost an order of magnitude higher than the relaxivities of many commercial contrast agents.

The syntheses of the **PLL** and **EA** dendrimers,[17] complexes **1**−**3**,[2] and complex **4**[20] have been reported previously. The conjugates were obtained by coupling the carboxylic acid functionalities of the dendrimer to the amine moiety of a Gd−TREN-bis-HOPO-TAM complex using carbodiimide as the coupling agent (Scheme 1). Because the gadolinium introduced into the reaction is already tightly bound as a highly stable complex, this precomplexation of the metal before conjugation reduces the possibility that free gadolinium ions might bind to nonspecific coordination sites. The conjugates were purified by precipitation into ether followed by aqueous size-exclusion chromatography (SEC) using PD-10 columns.

When **1** was coupled to the **EA** dendrimer (Scheme 1) inductively coupled plasma (ICP) and SEC measurements suggested an average loading of six gadolinium complexes per dendrimer. Despite extensive efforts, confirmation of this loading and determination of the nature of the gadolinium distribution using MALDI-TOF mass spectrometry could not be achieved. This gadolinium−**EA** dendrimer conjugate, **5**, has a relaxivity of 38.14 ± 0.02 mM^{-1} s^{-1} per gadolinium (228 mM^{-1} s^{-1} per dendrimer) at 37 °C and 60 MHz. This value compares very favorably to other relaxivities measured under clinically relevant conditions, which are typically in the range 3−5 mM^{-1} s^{-1} for current commercial agents (Figure 2), and even exceeds the

Scheme 1. Conjugation of Gd−TREN-bis(1-Me-3,2-HOPO)-TAM-ethylamine Complexes 1−3 to the EA Dendrimer (Conjugates 7 and 8 Were Synthesized under Identical Conditions)



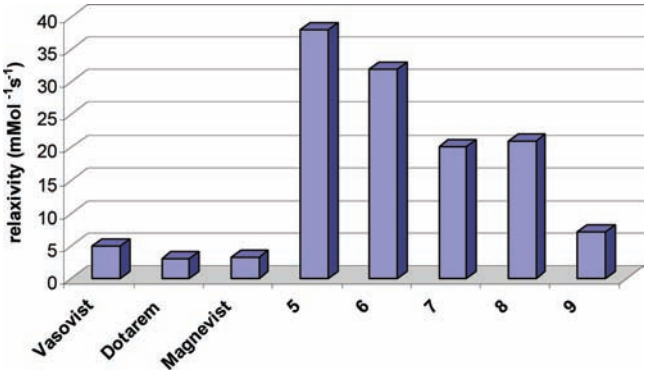


Figure 2. Comparison of the in vitro per-gadolinium relaxivities of several clinical Gd(III) contrast agents and the dendrimer contrast agents investigated. The values for conjugates **5**−**9** were measured at 37 °C and 60 MHz.

relaxivity of albumin-bound Vasovist, which can be in the range 35−45 mM^{-1} s^{-1}.[21]

We further investigated **6**, the conjugate of **2** with the **EA** dendrimer, because of its high thermodynamic stability. This small-molecule

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complex has a thermodynamic stability value 3 orders of magnitude larger than that of **1** or **3**.[2] This increased complex stability should reduce the amount of free gadolinium that can be leached from the conjugate in vivo, which may reduce toxicity concerns. However, because of the longer and more flexible linker, the molecular tumbling time and relaxivity were smaller than those of conjugate **5**, giving conjugate **6** a relaxivity of 31.9 ± 0.1 mM^{-1} s^{-1} per gadolinium. Although a lower complex loading per dendrimer was observed by ICP and SEC (2.8 complexes per dendrimer), conjugate **6** has the potential to be a safer option because of the higher thermodynamic stability of the gadolinium−chelate binding.

Given the success of the 1-Me-3,2-HOPO chelators, a variation on this moiety, the 1,2-HOPO chelator **4**, was evaluated. When **4** was conjugated to the **EA** dendrimer, per-gadolinium relaxivity values of 20.2 ± 0.6 mM^{-1} s^{-1} and an average loading of eight gadolinium complexes per dendrimer were obtained for the resulting conjugate **7**. In general the 1,2-HOPO complexes show lower relaxivities than their 1-Me-3,2-HOPO counterparts,[4,22] and this trend appears to be conserved upon conjugation to the dendrimer. These differences in relaxivities are believed to arise from differences in linker rigidity and the specific water coordination environment surrounding the gadolinium.

We also investigated ligand conjugation to the **PLL** dendrimer (see the Supporting Information for the structure), using chelate **1** to further test the versatility of this approach. **PLL** conjugate **8** has a relaxivity of 21.0 ± 0.6 mM^{-1} s^{-1} per gadolinium and an average loading of 4.5 gadoliniums per dendrimer. While this constitutes a significant increase over the small-molecule complex, it is still significantly lower than that obtained with the **EA** dendrimer. We attribute this decrease to greater internal hydrogen bonding between the inner-sphere water coordination sites of the gadolinium and the more hydrophilic amide-based **PLL** dendrimer core or to increased tumbling of the gadolinium(III) complexes caused by the less branched and sterically crowded core of this dendrimer. The decreased relaxivity observed with the **PLL** dendrimer, combined with the more favorable biodegradability of the **EA** dendrimer, suggests that the **EA** dendrimer is a superior platform for this application.

Finally, we investigated the conjugation of complex **3** with the **EA** dendrimer but found that the extended linker and extra primary amine resulted in a conjugate, **9**, with a relatively low relaxivity of 7.19 ± 0.07 mM^{-1} s^{-1} and an average loading of three gadoliniums per dendrimer. It seems possible that the lower relaxivity for this conjugate may arise from occupation of a gadolinium binding site by one of the free amines of the covalently bound chelate, which would effectively lower the q value of the ligand.

To evaluate the toxicity of these conjugates, cytotoxicity studies were carried out with HeLa cells for 72 h to determine their effect on cell viability using Gd-DTPA, **PLL**, and **EA** dendrimers as controls. While most MRI contrast agents are excreted within 24 h, the macromolecules may be excreted more slowly, and cytotoxicity testing was performed over a period of 72 h to reflect this increased residence time. None of the conjugates **5**−**9** exhibited evidence of cytotoxicity at a conjugate concentration of 1.0 mg/mL (see the Supporting Information), indicating that these contrast agents did not acquire short-term toxicity through their macromolecular conjugation.

Through conjugation to highly biocompatible and readily synthesized dendrimers, the relaxivities of HOPO-based, TREN-capped Gd(III) complexes were improved over those of commercial agents without compromising clinical relevance and safety. The conjugates presented here contain tightly bound gadolinium, exhibited relaxivities of up to 38 mM^{-1} s^{-1} under the clinically relevant conditions of 60 MHz and 37 °C, and were shown to be nontoxic to cells at millimolar concentrations.

■ ASSOCIATED CONTENT

Supporting Information. Conjugation procedures, characterization and cytotoxicity data, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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