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DTPA-bisamide-Based MR Sensor Agents for Peroxidase Imaging

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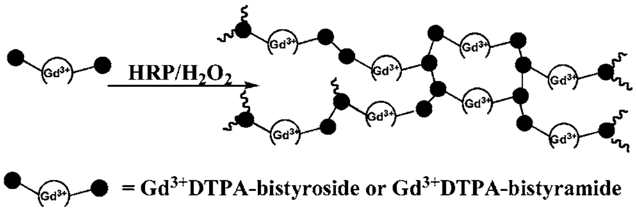
ABSTRACT



= Gd^{3+}DTPA-bistyroside or Gd^{3+}DTPA-bistyramide

The synthesis and some properties of two novel DTPA-bisamides are reported. These derivatives were designed as enzyme-activated contrast agents (CA) for magnetic resonance imaging. Both derivatives bear tyramido or 5-hydroxytryptamido groups that could be oligomerized in situ in the presence of peroxidase/H_2O_2 pair resulting in a net increase in longitudinal (R1) relaxivity.

Over the last two decades, magnetic resonance imaging (MRI) has become the leading tool for imaging fine details of anatomy as well as physiology (functional imaging). Due to high tissue contrast and excellent spatial resolution, Magnetic Resonance Imaging (MRI) is being used in many applications where optical methods fail to provide information due to light scattering and absorption by the tissue (usually, beyond the depth of 250 μm).[1]

In both clinical and research settings, MRI techniques benefit from the wide use of biocompatible Contrast Agents (CAs), which further enhance the image contrast. Currently, about 35% of all clinical MRI protocols include the use of several approved CAs.

The main application of CAs relies on the shortening of proton relaxation times (T_1 and T_2) of water molecules. The resultant subtle local MR signal changes can be easily detected giving rise to better signal-to-noise ratios and providing exact spatial locations if mapped versus noninfluenced water molecules in target tissue volume.[2] To be applicable for imaging in vivo, CAs have to exhibit high atomic relaxivities (r_{lp}) defined as the shortening of water proton relaxation rates in the presence of the CA normalized per concentration of the paramagnetic element. High relaxivites are essential to keep the dose of CA low while achieving high levels of contrast. In addition, pharmacokinetic properties of CAs have to be fine-tuned in order to achieve adequate in vivo retention and clearance times. In general, CAs, once administered, show the tendency to rapidly equilibrate between intravascular and interstitial compartments.[3] Fine-tuning of these properties should result in lower doses of paramagnetic metal and potential decrease in toxicity. Relaxivity increase and minimization of vascular permeability by using various macromolecular CAs have been goals of a large number of studies. In a broad sense, this research could be divided into two main categories. First, the increase in size has been achieved by covalent binding of CA to preformed macromolecules such as proteins, polypeptides,[4] dendrimers,[5] and graft-copolymers.[6] Second, tailored noncovalent association of CA with proteins, mainly

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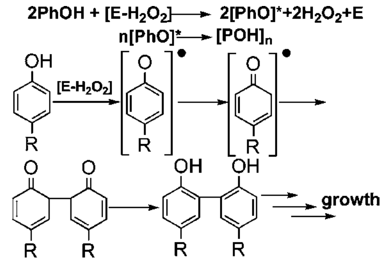
human serum albumin (HSA),[7] or micelle aggregation[8] has been described. However, theoretical limits of relaxivity increase are far from being reached.[9]

Despite all the previous efforts, the use of CAs to target specific biological events at the molecular level is still in its infancy due to the intrinsically low sensitivity of CAs to the presence of receptors and enzymes (biological targets).[10] The above problem has been tackled in several approaches that use monoclonal antibodies (mAbs) covalently attached to CAs.[11] In this regard, the use of mAbs has proven to be problematic since their affinity is usually negatively influenced by chemical modification with the CA. Specific enzymatic activation of CA precursors has also been reported. For example, enzyme-specific CA activation has been achieved by unmasking the gadolinium water coordination site upon galactosidase-mediated cleavage of a galactopyranose moiety that has the role of a water coordination site blocker.[12] Targeted probes for the detection of sialic acid[13] or sialyl LewisX[14] have also been reported. Recently, the use of HDL-like nanoparticles to image atherosclerotic plaque has been shown by Frias et al.[15]

We previously described GdDOTA-based paramagnetic substrates that undergo oxidoreductase-mediated polymerization.[16] The mechanism of polymerization is based on the oxidation of phenolic substrates in the presence of the enzyme and hydrogen peroxide to yield free radicals, which dimerize and/or give rise to oligomers (Scheme 1).[17] More recently, similar substrates were used for imaging myeloperoxidase activity in model systems.[18] The current communication describes the synthesis and preliminary characterization of two new bifunctional Gd-DTPA derivatives (MR sensor probes) that also polymerize in the presence of peroxidases, giving as a result an increase in relaxivity.

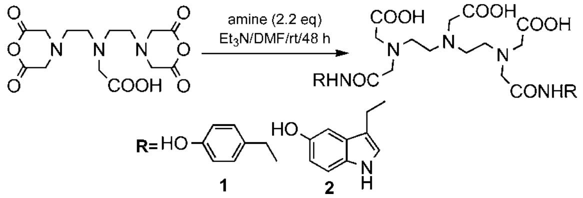
As an attempt to achieve improvements in enzyme-mediated relaxivity enhancement, we decided to link two groups prone to be oligomerized onto each chelate in order to achieve cross-linking and, therefore, higher rigidity of the final polymeric product. For the above purpose, DTPA was chosen as the basic chelate framework since its bismonomethylamide derivatives have shown no toxicity and are approved by the FDA for clinical use.

Scheme 1. General Scheme of Oxidoreductase-Mediated Reaction of Phenol Oligomerization



Scheme 2 shows the synthesis of compounds **1** and **2**. The synthesis was carried out starting from freshly prepared DTPA-bisanhydride, which was reacted with the corresponding amines in DMF in the presence of an excess of Et_3N. The products were isolated from the crude mixture by recrystallization from methanol and acetone and were used without further purification.

Scheme 2. Synthesis of Compounds **1** and **2**



The synthesis of corresponding Gd^{3+} complexes was complicated by oxidation of phenolic moieties. When compounds **1** or **2** were stirred either at room temperature or at 60 °C with an excess of Gd^{3+} salt (chloride or nitrate) at pH = 7, the solution turned dark with the formation of insoluble products. To avoid excessive oxidation, the chelation was performed in the presence of citric acid (1% w/w), resulting in a colorless reaction mixture. The attempts to precipitate the excess Gd^{3+} by raising the pH of the final solution to 10, at which Gd^{3+} forms insoluble hydroxide, were unsuccessful due to precipitation and decomposition of the complexes. The desired gadolinium complexes could be isolated in low yields after the HPLC purification of the untreated reaction mixture, though compound **1**Gd^{3+} always contained small amounts of its dimeric counterpart.

The relaxivities of various products were measured as described,[19] with a minimum of four different gadolinium complex concentrations ranging from 0.1 to 2.5 mM.

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(19) Values obtained for T_1 at different concentrations were fitted to the expression $(1/T_i) = (1/T_0) + r_{lp}[C]$, where [C] is the concentration expressed as mM, T_i is the relaxation time in s, and r_{lp} is the relaxivity expressed in $mM^{-1}\ s^{-1}$.

Horseradish peroxidase (HRP) that is widely used in immunochemistry was used in the proof-of-principle experiments. Oligomerization of compounds **1**Gd^{3+} and **2**Gd^{3+} was achieved by incubating the desired solution (0.5 mL) of monomer with an excess of 3% H_2O_2 (2 μL) and HRP (1 $\mu g \approx 4$ U) for 1 h at 40 °C. Both T_1 and T_2 were measured at two different field strengths: 0.47 and 1.5 T, this last one using a clinical magnet (1.5 T Signa, GE).

Relaxivity values of compounds **1**Gd^{3+} and **2**Gd^{3+} are shown in Table 1 under various tested conditions. While the first four rows show T_1 relaxivity values measured at 0.47 and at 1.5 T (clinical magnet field strength) of the monomers in deionized water (measured pH = 4.8) and in physiological conditions (in PBS: 10 mM sodium phosphate, 0.15 M NaCl, pH = 7.4), the last two rows include relaxivities of the macromolecular aggregates resulting from enzymatic catalysis in phosphate buffered solution.

Table 1. Relaxivities of the Different CAs

compd[a]	r_{1p}, $mM^{-1}\ s^{-1}$ (0.47 T, 40 °C)	r_{1p}, $mM^{-1}\ s^{-1}$ (1.5 T, 25 °C)
1Gd^{3+} (H_2O)	4.6	
2Gd^{3+} (H_2O)	4.5	
1Gd^{3+} (PBS)	4.3	5.3
2Gd^{3+} (PBS)	4.3	5.1
1Gd^{3+}/HRP	15.9	8.8
2Gd^{3+}/HRP	10.5	6.6[b]

[a] Parentheses define the solvent used. When HRP was employed, the solvent was PBS at pH = 7.4. H_2O pH = 4.8. [b] Fitting with three different concentrations due to precipitation at concentrations above 0.7mM. $r^2 = 0.98$. For all the rest $r^2 > 0.995$.

As expected, on the basis of the relaxivity values for DTPA or DTPA-bismonomethylamide at 0.47 T, the relaxivities of compounds **1**Gd^{3+} and **2**Gd^{3+} were in the range of 4.3 mM^{-1} s^{-1}. On the other hand, all relaxivites showed an increase when the above monomers were treated with the enzyme. In this regard, the use of HRP resulted in a 3.7-fold increase in [r_{1p}] for **1**Gd^{3+} and 2.4-fold increase in [r_{1p}] for **2**Gd^{3+} at 0.47 T and 40 °C. The increase in [r_{2p}] was in the same range for both compounds (not shown). When measurements were made on a 1.5 T clinical MRI unit at 25, a 1.7-fold increase in relaxivity was observed for compound **1**Gd^{3+}. The relaxivity of compound **2**Gd^{3+} could not be determined with high accuracy due to a linearity loss at high concentrations of **2**Gd^{3+} ($\sim$0.7 mM) when $1/T_i$ values were plotted against Gd concentration. Nevertheless, a minimum increase of 30% can be predicted from the obtained data (1.4-fold when concentrations below 0.5mM were used for fitting). Similar results were obtained when transverse relaxivities [r_{2p}] were measured (not shown). As suggested before, the above data can be explained by the formation of macromolecular aggregates with longer molecular rotational correlation times (τ_r) and, therefore, higher relaxivity. At this point, the actual degree of aggregation remains to be determined. The observed nonlinear dependence of relaxivity on concentration described above was associated with the formation of insoluble aggregates. To qualitatively prove the formation of such aggregates, the mixture of the enzyme and H_2O_2 was added to concentrated solutions (10^{-2} M) of compounds **1**Gd^{3+} and **2**Gd^{3+}. After the mixtures were incubated for a period of 2 h at 40 °C and then cooled to room temperature, we observed the initial formation of a gellike product that gradually associated and precipitated. Similar experiments with Eu^{3+} salts of compounds **1** and **2** demonstrated the formation of oligomeric products with an average oligomerization degree of 8 and 7, respectively, as shown by using MALDI-MS (see Supporting Information).

Further, a T_1-weighted image of a 96 well immunoanalysis plate fragment (4 × 4 wells) that contained **1**Gd^{3+} and **2**Gd^{3+} solutions was obtained to assess the contrast properties of the enzyme substrates in an MR imaging setup at clinically relevant field strengths (Figure 1A). Rows a_1 in Figure 1A contained (from left to right) 0.75, 0.5, 0.25, and 0.1 mM **1**Gd^{3+} (300 L) in DPBS, respectively. Samples in the row a_2 contained the same concentrations as above plus a fixed amount of HRP (4 U).[20] Rows a_3 and a_4 were prepared in the same manner as rows a_1 and a_2, respectively, but using compound **2**Gd^{3+}. The corresponding relaxivities measured are listed in Table 1.

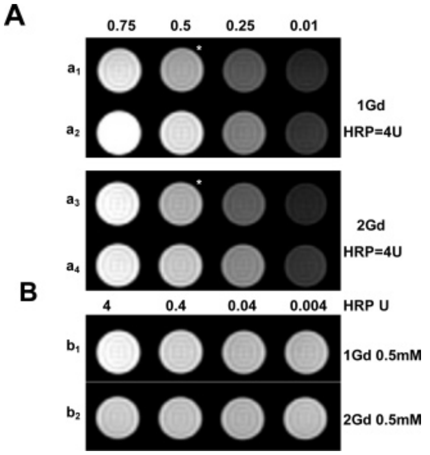

Figure 1. Well plate showing a T_1 map of compounds **1**Gd^{3+} and **2**Gd^{3+} acquired at 1.5 T. Concentrations in the wells were as indicated. Asterisks indicate wells with 0.5 mM substrate in the absence of HRP (concentration identical to wells in b).

Figure 1A clearly demonstrates predicted differences in MR signal intensity between the enzyme-containing and the enzyme-free substrate solutions, i.e., samples in the rows a_2 and a_4 show a net difference in signal when compared with the corresponding samples in rows a_1 and a_3 where no enzyme was used. The observed higher contrast is due to a 70 and 30% increase in relaxivity in the presence of peroxidase for compounds **1**Gd^{3+} and **2**Gd^{3+}, respectively.

To estimate the detection limit of these sensor substrates, a new well plate layout containing a fixed concentration of

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(20) Enzyme activity was measured as described by: Klebanoff, S. J.; Waltersdorph, A. M.; Rosen, H. *Methods Enzymol.* **1984**, *105*, 399−403.

1Gd^{3+} (0.5mM, row b_1) and **2**Gd^{3+} (0.5mM, row b_2) supplemented with different HRP amounts (from left to right: 4, 0.4, 0.04, 0.004 U) was imaged (Figure 1B). The results of the latter experiment are summarized in Table 2.

Table 2. HRP Effect over T_1 at $c = 0.5$ mM for **1**Gd^{3+} and **2**Gd^{3+}

HRP (U)	T_1 **1**Gd^{3+} (ms)	T_1 **2**Gd^{3+} (ms)
0	316	339
4	211	271
4 10-1	229	278
4 10-2	274	303
4 10-3	321	331

The use of 4 U/mL of enzyme yielded a decrease in T_1 by 50 and 25% for compounds **1**Gd^{3+} and **2**Gd^{3+}, respectively. A detectable decrease could be seen at enzyme specific activity levels as low as 0.04 U/mL. In this case, the decrease was measured to be 15% for compound **1**Gd^{3+} and 12% for compound **2**Gd^{3+}. The use of lower enzyme activities did not yield any further improvement.

Besides the expected variations in measured relaxivity associated with differences in field strengths, the differences imaged thus far could be explained in terms of different degrees of polymerization/cross-linking due to variations in enzymatic activity and different stability of the intermediate radicals (intraradical association kinetics is predicted to be diffusion limited).[21] These differences in aggregation also led to different solubility patterns along the different wells. This resulted in a loss of linearity of the dependence between relaxation time and concentration in row a_4 where turbidity could be observed (well 1, concn = 0.75 mM, 4 U HRP) during the course of the measurement. Those inhomogeneities proved to be more striking when T_2-weighted images were acquired (not shown) since paramagnetic precipitates cause strong magnetic field inhomogeneities. Nonetheless, the differences in enzymatic activity were important since they suggest the feasibility of specific Gd-based probe development for various oxidoreductases. These probes could have potential clinical applications since the enzyme activity could be clearly detected using clinical strength magnets.

In summary, we demonstrated the possibility of using oxidoreductases to convert monomeric, bifunctional CAs into oligomeric products with increased relaxivity. The relaxivities obtained thus far can be compared to several other macromolecular CAs (e.g., dendrimers, peptide-based or protein-bound chelates) with the difference that those relaxivities are generated in situ by enzyme-mediated activation, which could lead to in vivo detection of such enzymes. The relaxivity values were as high as 15.9 mM^{-1} s^{-1} with MR signal enhancements in the range of 3.7−1.7-fold. Thus, in the presence of equal HRP concentrations, the above effects were more prominent than in the case of Gd salts bearing only one oligomerization unit.[16,18] Furthermore, compounds **1**Gd and **2**Gd were obtained through a one-step synthesis procedure from readily available precursors as opposed to monoderivatives that were obtained through a more complex synthesis requiring multiple protection and deprotection steps.

Detection limits of 0.04 U/mL could also be established. Those values are sufficiently high to encourage the development of the substrates for biomedical applications.

To better define the scope of the new MR sensor probes, experiments that focus on kinetic parameters and detection limits for in vivo applications are currently under way. Specifically, these compounds are being essayed for sensing other peroxidases such as myeloperoxidase (MPO) as reported in ref 18. This enzyme is secreted by neutrophils and macrophages in response to inflammation, and therefore its monitoring would enable the assessment of inflammatory processes associated with atherosclerosis,[22] among others. Preliminary results obtained so far with **2**Gd^{3+} at pH = 7.4 (PBS buffer), have shown a minimum relaxivity increase of 40% at 0.47 T.

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Supporting Information Available: Synthesis and characterization of compounds **1** and **2** and imaging parameters and MALDI of HRP reaction products with compounds **1**Eu^{3+} and **2**Eu^{3+} upon reaction with HRP. This material is available free of charge via the Internet at http://pubs.acs.org.

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