A Calcium-Sensitive Magnetic Resonance Imaging Contrast Agent

Wen-hong Li, Scott E. Fraser, and Thomas J. Meade*

Division of Biology and the Beckman Institute
California Institute of Technology
Pasadena, California 91125

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Ca^{2+} is an important intracellular secondary messenger of signal transduction.[1] Changes in the cytosolic concentration of Ca^{2+} trigger changes in cellular metabolism and are responsible for cell signaling and regulation.[2] Advances in optical microscopy techniques and improvements in fluorescent dyes capable of measuring Ca^{2+} concentration have added greatly to the understanding of the critical role this ion plays in cell and neurobiology.[3] However, a fundamental limitation of light-based microscope imaging techniques employing dyes or fluorochromes is that they produce toxic photobleaching byproducts and are limited by light scattering to those cells within 100 μm of the surface. Magnetic resonance imaging (MRI) of biological structures provides an alternative to light-based microscopy that can circumvent these limitations. Recent work in this area has demonstrated the feasibility of true three-dimensional MR imaging at cellular resolution (∼10 μm).[4]

As part of our efforts to study cell signaling and regulation in intact animals, we are developing MRI contrast agents that provide information about physiological signals and biochemical events. To this end, we have prepared and tested a class of enzymatically activated MRI contrast agents that conditionally enhance image intensity.[5] Here, we report the first MRI contrast agent, DOPTA−Gd (Figure 1), whose relaxivity is selectively modulated by Ca^{2+} concentration.[6] DOPTA−Gd has a Ca^{2+} dissociation constant of 0.96 μM, and the relaxivity of the complex increases approximately 80% when Ca^{2+} is added to a Ca^{2+}-free solution.

The most abundant molecular species in biological tissues is water. It is the quantum mechanical "spin" of the water proton nuclei that ultimately gives rise to the signal in all imaging experiments. MRI agents enhance the intrinsic differences in the T_1 (spin−lattice) and T_2 (spin−spin) relaxation rates. The class of contrast agents referred to as T_1 agents accelerate the T_1 relaxation rate, increasing the signal from nearby water protons and making a voxel appear "brighter" in the resulting image. The increase in relaxation rate is due, in part, to the direct interaction of water molecules (inner sphere) with the unpaired electrons of a paramagnetic metal ion. The lanthanide ion, Gd^{3+}, is frequently chosen for MRI contrast agents because it has a very high magnetic moment ($\mu^2 = 63\ \mu_B^2$), and a symmetric electronic ground state, $^8S_{7/2}$. The Gd^{3+} aqua ion is toxic and hence is chelated to a ligand in order to reduce toxicity. Typically, eight of the nine available Gd^{3+} coordination sites are bound by the chelate, leaving one site available for an inner sphere water molecule.[5,7]

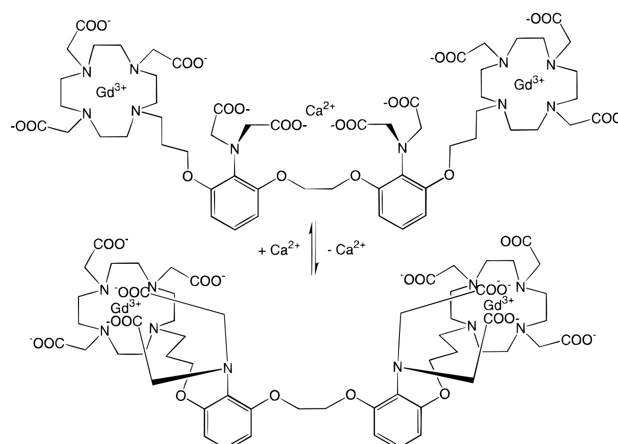

Figure 1. Schematic of DOPTA−Gd representing the proposed conformational dependence of the structure in the presence and absence of Ca^{2+}.

This new class of MRI agents modulates access of water to a chelated Gd^{3+} ion in the presence and absence of Ca^{2+}. The design of the agent is based on the synthesis and characterization of several model systems that ultimately led to the macrocyclic dimer shown in Figure 1. 1,2-Bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) binds Ca^{2+} with a 10^5-fold selectivity versus the divalent metal ion Mg^{2+} and is relatively insensitive to pH fluctuations at physiological conditions (>6.8).[8] 1,4,7-Tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane (DO3A) chelates lanthanides with high affinity to form a thermodynamically stable and kinetically inert complex.[9] DOPTA−Gd (Figure 1) was designed to possess two limiting conformational states with respect to calcium concentration ([Ca^{2+}]). In the absence of Ca^{2+}, the aromatic iminoacetates of BAPTA interact with Gd^{3+} through ionic attractions. In the presence of Ca^{2+}, the aromatic iminoacetates of BAPTA will rearrange to bind Ca^{2+}, thereby allowing water to bind directly to Gd^{3+}.

On the basis of complexes synthesized as part of model studies, a propyl linker was chosen to covalently connect DO3A to BAPTA.[10] The propyl linker places the aromatic iminoacetates of BAPTA in the proximity of the chelated Gd^{3+}. Therefore, the aromatic iminoacetates shield the Gd^{3+} ion from water when the Ca^{2+} concentration is low. Upon binding Ca^{2+}, the complex undergoes reorganization that exposes the Gd^{3+} ion to bulk water, thereby changing the relaxivity from weak to strong.

DOPTA−Gd was synthesized from nitroresorcinol in 8 steps (Figure 2). The monoalkylated nitroresorcinol **1** was dimerized with 1,2-dibromoethane. The free hydroxyls of **4** were converted into dibromides **5**. Excess cyclen was used to minimize the formation of the bisalkylated product during the synthesis of **6**.

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(10) Model complexes with varying linker lengths were prepared to optimize the modulation of water access to the Gd^{3+} ion based on data from T_1 and luminescence lifetime measurements.

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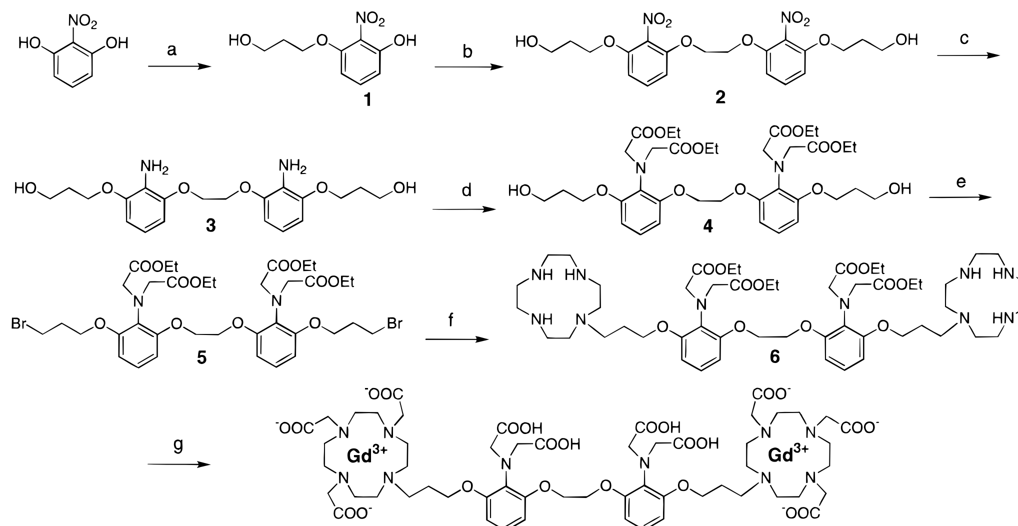

Figure 2. Synthetic scheme of DOPTA−Gd. (a) NaOH, DMF, 3-bromopropanol, 42%. (b) K_2CO_3, DMF, 1,2-dibromoethane, 55%. (c) Pd/C (10%), EtOH/EtOAc, H_2, 1 atm. (d) Bromoethyl acetate, DIEA, CH_3CN, 61% for 2 steps. (e) CBr_4, PPh_3, Et_2O, 60%. (f) Cyclen (5 equiv), $CHCl_3$, 80%. (g) 1. Bromoacetate, NaOH, pH > 10, 82%; 2. $GdCl_3$, NaOH, pH 5−6, 81%.

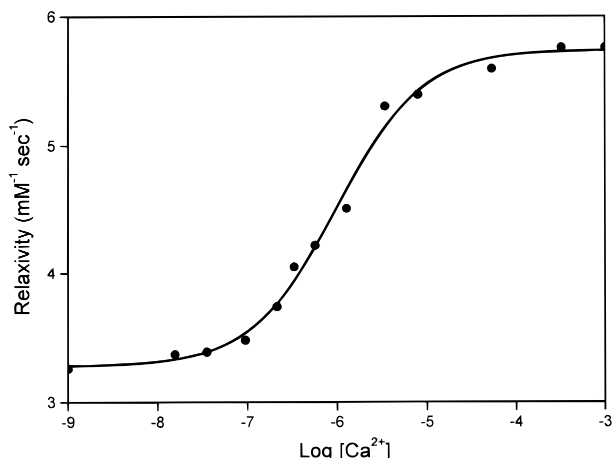

Figure 3. Relaxivity measurement of DOPTA−Gd as a function of free [Ca^{2+}].[11] The fitted curve corresponds to the apparent dissociation constant = 0.96 μM and Hill coefficient = 0.92.

The remaining secondary amines were alkylated under basic conditions that concomitantly hydrolyzed the aromatic imino ethyl diacetates. The final Gd^{3+} complex was formed in good yield under weakly acidic conditions.

The effect of [Ca^{2+}] on the relaxivity of DOPTA−Gd was assessed by T_1 measurements.[11] The relaxivity of DOPTA−Gd in Ca^{2+}-free buffer was 3.26 mM^{-1} sec^{-1} and increased with increasing [Ca^{2+}]. The change in relaxivity is most striking in the [Ca^{2+}] range of 0.1 to 10 μM and levels off at higher levels of [Ca^{2+}], reaching a maximum of 5.76 mM^{-1} sec^{-1} (Figure 3). Hill-plot analysis of the measured relaxivities at varying [Ca^{2+}] results in a dissociation constant of 0.96 μM (Hill coefficient of 0.92). These results reveal a 1:1 stoichiometry of binding.[8]

The increase in observed relaxivity of DOPTA−Gd (∼80%) that is induced by an increase in [Ca^{2+}] corresponds to a 80% relaxivity change of each Gd^{3+} unit and is significantly higher than our previously reported enzyme−reporter class of agents (E−Gad: 20% change of T_1 at a [Gd^{3+}] of 2 mM after enzyme cleavage).[5] Since BAPTA binds Ca^{2+} with a much higher selectivity than Mg^{2+}, the relaxivity of DOPTA−Gd should be relatively insensitive to [Mg^{2+}] change. Indeed, increasing Mg^{2+} concentration from 0 to 10 mM changed the relaxivity of DOPTA−Gd less than 8%.[12] Intracellular [Mg^{2+}] is approximately 1 mM, and its fluctuation is less dynamic than [Ca^{2+}]; therefore, interference on the [Ca^{2+}] measurements by DOPTA−Gd should be minimal.

The effect of H^+ on the observed relaxivity of DOPTA−Gd was also tested. Changing the pH from 6.80 to 7.40 (in 0.2 pH unit steps) changed the measured T_1 of DOPTA−Gd by less than 3% (in the presence or absence of Ca^{2+}).[13] Therefore, within physiological pH ranges, H^+ should not interfere with the relaxivity of the complex with respect to [Ca^{2+}].

In summary, we have synthesized a MRI contrast agent where the relaxivity of the complex is controlled by the presence or absence of the divalent ion Ca^{2+}. By structurally modulating inner-sphere access of water to a chelated Gd^{3+} ion we observe a substantial change in T_1 upon the addition of Ca^{2+}. Importantly, the agent is selective for binding Ca^{2+} ions versus Mg^{2+} and H^+. An immediate application of this agent is to study the change in cellular calcium activity during embryogenesis. The agent can be conveniently microinjected inside cells at an early developmental stage, and subsequent cell movements and calcium fluctuations during the development can be monitored over long periods of time. These investigations may help to resolve current uncertainties concerning cellular Ca^{2+} activities of interior cell layers not accessible to light microscopy. Finally, this new class of contrast agent offers a large and reliable change in relaxivity upon exposure to Ca^{2+}. These results serve as an ideal template for the future design of biochemically activated MRI contrast agents.

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(11) [Ca^{2+}] was controlled by Ca^{2+}/EGTA and Ca^{2+}/HEEDTA systems below 10^{-5} M free Ca^{2+} and unbuffered Ca^{2+} above.[8] The buffers contained 100 mM KCl, 10 mM KMOPS, pH 7.20, 20 mM of EGTA or HEEDTA. The T_1 was measured by using the standard inversion−recovery procedure (Bruker AMX 500, 25 °C).[5] The relaxivity of DOPTA−Gd was determined from the slope of the plot of $1/T_1$ vs [DOPTA−Gd] (0, 0.2, 0.3, 0.4 mM).

(12) The longitudinal relaxation rate of DOPTA−Gd (0.3 mM) was measured in a buffer containing 130 mM KCl, 10 mM MOPS and 1 mM EGTA, pH 7.20, 25 °C. Mg^{2+} concentration was adjusted to 2, 5, and 10 mM using the reported procedures.[8a] The relaxivity (3.23 mM^{-1} sec^{-1}, 0 Mg^{2+}; 3.30 mM^{-1} sec^{-1}, 2 mM Mg^{2+}; 3.39 mM^{-1} sec^{-1}, 5 mM Mg^{2+}; 3.47 mM^{-1} sec^{-1}, 10 mM Mg^{2+}) was calculated from the T_1 after subtracting the longitudinal relaxation rate in the absence of DOPTA−Gd.

(13) The T_1 was measured in buffers containing 100 mM KCl, 5 mM MOPS, 0.3 mM DOPTA−Gd with either 2 mM EGTA or 1 mM $CaCl_2$ at 25 °C. The pH was varied from 6.80 to 7.40 in 0.2 pH unit steps with HCl or KOH.