TOWARDS THE DEVELOPMENT OF GADOLINIUM FUNCTIONAL MAGNETIC RESONANCE IMAGING (fMRI) CONTRAST AGENTS: CONTROLLING DISTRIBUTION AND RELAXIVITY

Reported by Nathan C. Ackroyd December 5, 2002

INTRODUCTION

In 1973, Lauterbur introduced a method of localizing the source of a magnetic resonance signal using orthogonal magnetic fields.[1] Using related methods, Kumar et al. published an image of a human finger in 1975,[2] and in 1977, imaging was extended to cross-sections of the wrist[3] and chest.[4] The use of paramagnetic ions to enhance contrast was first introduced in 1982 by Hinshaw and co-workers,[5,6] and in 1984 the gadolinium chelate Na_2[Gd(DTPA)] (Figure I) was used for imaging human cerebral tumors.[7] Currently, all Paramagnetic Contrast Agents (PCAs) approved for clinical use in Magnetic Resonance Imaging (MRI) are derivatives of the open chain DTPA (diethylenetriaminepentaacetic acid) or the macrocyclic chelate DOTA (Figure II) (tetraazocyclododecanetetraacetic acid). In addition, clinically approved PCAs exhibit either no tissue selectivity, or show only selective uptake into hepatocyte cells as they are cleared by the liver. Imaging with these non-selective PCAs is done on a time- or pulse sequence-dependent basis, which provides images of structure at the tissue level. In contrast, functional MRI (fMRI) seeks to image biological function at a molecular level and requires the introduction of selectivity in distribution and activation. This review will cover several approaches for introducing selectivity into MRI, which has traditionally been a non-selective method for imaging.

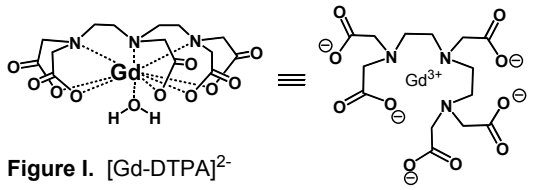

Figure I. [Gd-DTPA]$^{2-}$

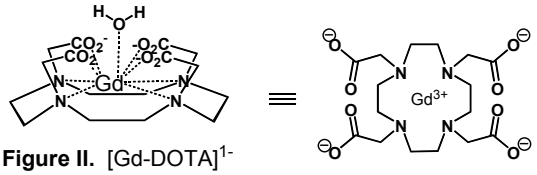

Figure II. [Gd-DOTA]$^{1-}$

PRINCIPLES OF fMRI: RELAXATION, CONTRAST AGENTS AND SELECTIVITY[8]

To convert a non-directional magnetic field into an image, orthogonal magnetic field gradients are used. Because the Larmor frequency of a nucleus in a magnetic field is proportional to the field strength, a field gradient leads to a systematic variation in Larmor frequency of protons throughout the sample space. Protons can thus be differentiated according to excitation, relaxation and phase differences. Three orthogonal gradients allow for signal localization to a 3-D space, volume element (voxel).

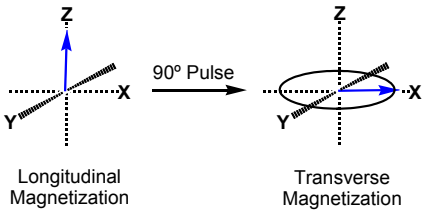

Figure III. Net Magnetization Vectors

The precession of nuclear magnetic moments about the Z-axis of a static magnetic field, such as used in MRI, leads to a sum magnetization that lies along the Z axis. This is referred to as *longitudinal* magnetization (Figure III). During an excitation pulse, gain of phase coherence and spin flip of nuclear magnetic moments lead to a rotation of net magnetization towards the XY plane, which is referred to as *transverse* magnetization. A 90° excitation pulse gives maximum transverse magnetization. Relaxation refers to the return to the equilibrium state; thus, longitudinal relaxation (T1) is re-growth of longitudinal magnetization, and transverse relaxation (T2) is disappearance of transverse magnetization, each relaxation time T being defined as the time required for a return to 63% of the equilibrium value and 1/T being the rate constant for this first order relaxation process.

Relaxation occurs by two main processes. First, T1 relaxation occurs by transfer of energy to the surrounding structures, or lattice. The efficiency of this energy transfer is dependent on the rotational correlation time (τ_R) or the time between successive positional reorientations. Thus, slower rotating molecules, such as water bound to a macromolecule, exhibit faster T1 relaxation. Second, T2 relaxation is affected additionally by the loss of phase coherence caused both by entropy and fluctuations in local magnetic environment that alter the precession rate. As a result, 1/T2 is always faster than 1/T1.

In MRI, images can be either T1 or T2 weighted. If the repetition time between excitation pulses (TR) is equal to T1 or less, incomplete relaxation occurs before subsequent excitation. Hence, faster relaxing nuclei have more complete longitudinal magnetization, and exhibit a larger transverse magnetization following subsequent excitation. Because signal is obtained from magnetization in the XY plane, faster relaxing nuclei appear brighter in a T1 weighted image. If the delay between excitation and detection (TE) is longer than T2, faster relaxing nuclei have less transverse magnetization, resulting in a darker signal in T2 weighted images. Thus, at a first approximation, a T2 weighted image appears as a negative of T1.

Gadolinium-based Paramagnetic Contrast Agents (PCAs) consist of a Gd^{3+} ion bound to an octadentate chelate to reduce the toxicity of the heavy metal. Gadolinium exhibits nine-coordinate geometry, so water binds to the one site left open by the chelate. Gd PCAs reduce the relaxation time of water through interactions between the unpaired electrons on the metal and metal-bonded (inner-sphere) and hydrogen-bonded (outer sphere) water molecules.

The first PCA approved for clinical use was $[Gd(DTPA)]^{2-}$, or Magnevist®. This hydrophilic chelate diffuses readily across vascular membranes; thus, imaging of the arteries must be accomplished within 20-30 s post injection. The blood-pool (arteries and veins) is imaged from 1-2 min post injection, after which the PCA has diffused into the extracellular space. Imaging the extracellular space can take place over several minutes to half and hour, as the PCA is filtered and excreted via the kidneys. Such time-dependent imaging is known as dynamic-contrast-enhanced (DCE-MRI) and provides images of differences such as volume and permeability.[8,9] In contrast to this non-specific imaging, functional imaging (fMRI) attempts to image biological function at the microscopic or molecular level, such as molecular displacements or metabolic changes, and requires the introduction of selectivity.[10] A number of approaches exist to introduce selectivity in fMRI agents. These include reversible plasma protein binding, and enzyme activated Contrast Agents.

MACROMOLECULE BINDING PCAs: LOCALIZATION AND ACTIVATION

As discussed above, slowing the rotation time (τ_R) of an excited molecule by having it bind to a macromolecule leads to faster T1 relaxation. When such binding is reversible, this effect is known as Receptor Induced Magnetization Enhancement (RIME). In addition to decreasing T1, macromolecule binding can control the tissue distribution of a PCA. A rational approach to achieve effective RIME is needed, however, because simply increasing the hydrophobicity of a chelate has generally led to increased uptake by hepatocytes and excretion through the liver, rather than increased non-specific binding to serum proteins.[11,12] The *ex vivo* covalent attachment of a small PCA to a macromolecule such as Bovine Serum Albumin or polylysine leads to favorable blood-pool imaging, but poor excretion properties.[13]

To avoid these problems, Lauffer and co-workers have designed MS-325 (Figure IV), a PCA that binds Human Serum Albumin (HSA) reversibly *in vivo*. MS-325 binds to several sites on HSA, using an amphiphilic phosphodiester to mimic the binding of the Heme degradation product bilirubin. The reversible binding of this agent couples the blood-pool retention of a macromolecule with the excellent excretion properties of small molecules.[14,15]

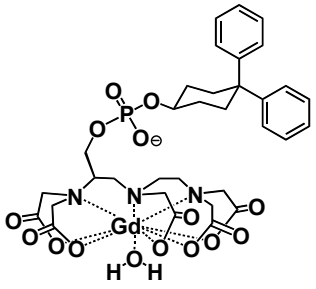

Figure IV. MS-325

In another example, Anelli and co-workers have used a sulfonamide-derivatized DTPA (Gd-DTPA-SA) to bind specifically to Carbonic Anhydrase (CA).[16] The rationale for this approach arises from the well documented inhibition of CA by sulfonamides.[17] Gd-DTPA-SA binds to erythrocyte CA

in vitro and increases the observed $1/T_1$ value from 3.5 s^{-1} to 25 s^{-1}. However, binding to CA on erythrocytes is insufficient to localize the PCA in the blood-pool *in vivo*.

MS-325 effectively localizes to the blood-pool due to changes in the polarity of the chelate. The example of Gd-DTPA-SA could also be categorized as a pendant moiety functionalized chelate because it is an attached functional group that promotes binding, rather than a general property such as hydrophobicity.

ENZYME ACTIVATED CONTRAST AGENTS

Previously discussed methods for controlling selectivity in fMRI involve the selective delivery of a PCA to a cell or a tissue. Another approach is universal delivery coupled with selective activation of a PCA from a low relaxivity to high relaxivity state. This can be accomplished by initially blocking an open coordination site with an enzyme-cleavable moiety. Subsequently, in the presence of the target enzyme, a coordination site is opened, allowing water to bind, thus increasing the relaxivity of the system. Pioneering work by Meade and co-workers has demonstrated the utility of this approach in the development of a MRI system to image reporter gene expression.[18-20]

The introduction of a gene of interest into a cell is often done in combination with a reporter gene, a gene that encodes an easily observable product. The presence of the observable gene product indicates, or reports, expression of the gene of interest. One common reporter gene is *Lac-Z*, the gene encoding β-galactosidase. Treatment of cells expressing β-galactosidase with X-gal (5-bromo-4-chloro-3-indoyl—D-galactopyranoside) results in the production of a blue dye, which is detected optically.[21]

Meade and co-workers synthesized a hydroxyethyl Gd-DOTA derivative with a β-Galactopyranose moiety (EGad) that blocks the ninth coordination site of Gd^{3+}.[19] The blocking moiety was removed by exposure to β-galactosidase (β-Gal), causing a 20% decrease in T1 over 20 h at 37 ºC. Though this demonstrated the feasibility of the blocking moiety approach, high contrast between activated and control samples *in vitro* was only observed using an inversion-recovery pulse sequence, a method that leads to long acquisition times and poor S/N ratios.

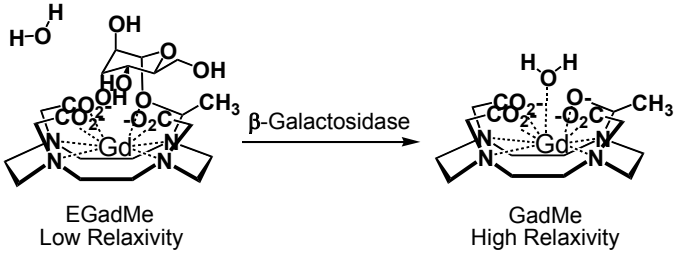

Figure V. Enzyme Acitvation of EGadMe

Subsequent change of the hydroxyethyl-DO3A chelate of Egad to a hydroxypropyl (EGadMe) decreased the flexibility of the blocking moiety (Figure V).[18] This additional rigidity led to a decrease in the relaxivity of the uncleaved complex; thus, cleavage resulted in a 2-fold decrease in T1. *In vivo* imaging of *X.*

laevis embryos demonstrates the use of EGadMe in a living system. At the two-cell stage of development, both cells were injected with EGadMe, while one cell was also injected with mRNA coding for β-Gal and nuclear-localized green fluorescent protein (nGFP). Subsequent cell division produces daughter cells leading roughly to the left and right half of the embryo. A MRI signal enhancement of 57% was observed between the right half, which expressed β-Gal, and left half, which did not. High correlation was observed between MR images and optical observation using nGFP and X-gal staining. The advantage of MRI observation of reporter gene expression over methods using visible light is evident when MR images are compared to those obtained from nGFP and staining with X-Gal. Only the MR image allows for the depth of detection needed to show β-galactosidase expression in the interior of the head; imaging with visible light is compromised by scattering from the overlying tissue.

A number of factors still preclude the use of enzyme-activated PCAs in humans to image disease states associated with high levels of enzyme. First, in many applications, MRI agents capable of entering the cell are needed for exposure to enzymes. Use of EGadMe in live animals currently requires microinjection directly into cells at an early stage of development. Second, clinical application of MRI is typically done at field-strengths below 1.5 T (64 MHz ^{1}H Larmor Frequency). Meade and co-workers used a field-strength of 11.7 T (506 MHz ^{1}H Larmor Frequency), presumably to ensure sufficient signal intensity.

One solution to the need for microinjection of Contrast Agent involves the use of Membrane Translocation Sequence Peptides. Membrane Translocation Signal (MTS) peptides have been found that promote cellular uptake.[18,22-28] To date, only one of these, the Tat peptide, has found use in potential Gd-based imaging applications.[29,30]

In vitro experiments were done in which a Tat(48-60) peptide, cross-linked to a Gd-DOTA through the C-terminal Lys (Figure VI), was incorporated in cells from the HeLa cell line, mouse lymphocytes, and human natural killer cells. The chelate is retained inside the cell for several days, with no increased rate of cell death (20% morbidity rate) compared to controls after 5 days.

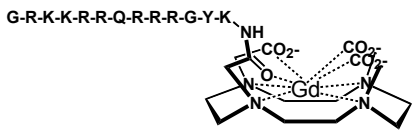

Figure VI. [Tat-DOTA-Gd]

A COMBINATION APPROACH

Examples that illustrate a combination of the strategies discussed thus far involve a chelate bearing a moiety that promotes binding to a macromolecule, and an enzyme-cleavable moiety that inhibits binding (Figure VII). Lauffer and co-workers have investigated the use of chelates tagged with an amphiphilic moiety that promotes HSA binding when a second hydrophilic group is cleaved enzymatically.[31]

In one example, these workers engineered a substituted DTPA chelate bearing aromatic groups to promote HSA binding, and a trilysine tripeptide that reduces HSA affinity. Lysine residues are removed sequentially from **1** and **5** by carboxypeptidase B, or thrombin-activatable fibrinolysis inhibitor (TAFI). This enzyme cleaves the C-terminal lysine from fibrin binding sites to inhibit blood clot degradation and has been implicated in thrombotic disease.

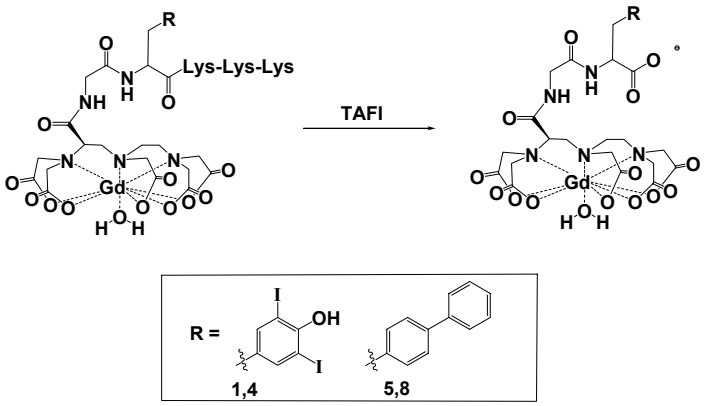

Figure VII. Enzyme Agents with Increased Binding to HSA

In vitro studies show that **4** has a 2.2 fold increase in 1/T over **1** at 37 °C. Similarly, the $1/T_1$ value of **8** is 2.7 times greater than **5**. Studies of the rates of lysine removal at physiological concentrations of TAFI (75 nM) show that **1** is incompletely converted to **4** after 30 min, with a 33% increase in $1/T_1$. Longer reaction times led to more complete hydrolysis, but with only minimal gains in $1/T_1$. In contrast, the $1/T_1$ increase shown by the conversion of **5** to **8** appeared to require the complete removal of lysine. After 60 min, **8** comprised 44% of all species present, while $1/T_1$ had increased by 26%. The incomplete conversion of **5** to **8** results from the autoinactivation of TAFI which is competitive with cleavage of the third lysine.

A modest increase of 26-33% in $1/T_1$ is not sufficient to provide satisfactory imaging, and further optimization is required before successful use *in vivo* is possible. However, another example by Lauffer et al., in which a phosphate inhibits binding of the PCA to HSA, further demonstrates the potential of this approach (Figure VIII). When the hydrophilic phosphate is cleaved by phosphatase, the resulting aromatic alcohol demonstrates sufficient amphiphilicity to bind to HSA with increase affinity. The slower τ_R of the large complex increases $1/T_1$ by 70%.[32,33] Further studies are underway to develop enzyme activatable HSA binding PCAs for study using MatrixMetalloProteinases (MMP), observed in some cancers, and elastase, observed in inflammation.

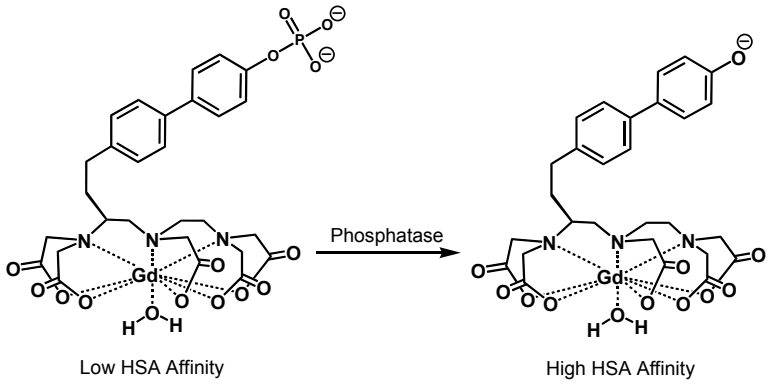

Figure VIII. Phosphatase Activated Contrast Agent

CONCLUSION

Advances in design strategies of PCAs for use in enzyme activated fMRI has led to significant increases in the selectivity of tissue distribution and relaxivity as a function of environment. However, these advances are limited to select examples and have yet to be broadly applied. This is due, in part, to the highly developed pulse sequences that have provided high quality structure-based imaging using clinically approved PCAs, such as Magnevist®. Attempts to image based on increased enzyme activity requires concurrent advances in cell penetration and tissue selectivity, such as provided by MTS peptides and albumin binding chelates, and suffers from lack of sensitivity due to the difficulty of generating sufficient concentrations of enzyme products. Of the factors contributing to decreased relaxation time of water in the presence of a Contrast Agent and the subsequent increase in signal intensity of the image, the greatest effect is due to decreased rotation time. Therefore, enzyme activation leading to increased binding to macromolecules has the greatest potential for overcoming sensitivity issues. In addition, for clinical use the rate of enzymatic cleavage must be rapid. The conversion of EGad and related Contrast Agents is prohibitively slow, perhaps due to the globular nature of these Agents, and the resulting poor fit in the active site of β-Galactosidase. Cleavage of blocking moieties further removed from the bulky chelate, such as those involved in contrast agents that bind to macromolecules, also appears to overcome this limitation. In view of these requirements for *in vivo* use of enzyme activated contrast agents, the combination of strategies developed by Lauffer, et al. appear to have greater potential for detection of disease states associated with increased enzyme activity than the strategy of blocking access of water to gadolinium, as developed by Meade.

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