NORTHWESTERN UNIVERSITY

Towards Detection of Intracellular Calcium Flux by Magnetic Resonance Imaging.

A DISSERTATION

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Field of Chemistry

By
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Abstract

Magnetic resonance imaging is a powerful research tool for studying anatomical processes in intact large organisms, as it is capable of generating high resolution images with unlimited penetration depth and excellent soft-tissue contrast. However one of the challenges faced by those seeking to use the technique to answer biochemical questions, is the inherent low sensitivity towards responsive probes. This requires careful selection of the model system and pathways to be investigated. The work in this thesis was aimed at expanding the research space accessible to magnetic resonance imaging methods to include detection of activity in the central nervous system. Specifically, visualization of intracellular calcium flux. This could provide a means for studying fundamental signaling information in deep brain regions inaccessible to other imaging techniques. Towards this aim, an existing calcium-sensitive agent was evaluated in two unique animal models: cannulation in rats and microinjection in mice. Learning from the shortcomings of this agent in vivo, new calcium responsive agents designed to have enhanced cellular permeability were synthesized and subjected to preliminary characterization.
Acknowledgements

There are so many people that have helped me to get to this point. First and foremost though is my family. I am so thankful that you are in my life and I don’t know where I would be without all of you. To my mom and dad: thanks for the unconditional love and support throughout the years. I love you both so much. Nate: I’m looking forward to taking some cool trips in the future and spending more time in SC. Every time we talk I’m so happy about all the great stuff you’ve got going on these days.

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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>MI</td>
<td>molecular imaging</td>
</tr>
<tr>
<td>NIR</td>
<td>near infrared</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>SPECT</td>
<td>single photon emission computed tomography</td>
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<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>RF</td>
<td>radiofrequency</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
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<tr>
<td>fMRI</td>
<td>functional magnetic resonance imaging</td>
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<tr>
<td>BOLD</td>
<td>blood oxygen-level-depend</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CA</td>
<td>contrast agent</td>
</tr>
<tr>
<td>SBM</td>
<td>Solomon-Bloembergen-Morgan</td>
</tr>
<tr>
<td>IS</td>
<td>inner sphere</td>
</tr>
<tr>
<td>SS</td>
<td>second sphere</td>
</tr>
<tr>
<td>OS</td>
<td>outer sphere</td>
</tr>
<tr>
<td>NMRD</td>
<td>nuclear magnetic relaxation dispersion</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>paraCEST</td>
<td>paramagnetic chemical exchange saturation transfer</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet-visual</td>
</tr>
<tr>
<td>AM</td>
<td>acetoxymethyl</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>MRS</td>
<td>magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>MEMRI</td>
<td>manganese-enhanced magnetic resonance imaging</td>
</tr>
<tr>
<td>KA</td>
<td>kainic acid</td>
</tr>
<tr>
<td>CA3</td>
<td>third cornu ammonis</td>
</tr>
<tr>
<td>TE</td>
<td>echo time</td>
</tr>
<tr>
<td>TR</td>
<td>repetition time</td>
</tr>
<tr>
<td>GRE</td>
<td>gradient echo</td>
</tr>
<tr>
<td>SI</td>
<td>signal intensity</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>ESI MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix assisted laser desorption/ionization</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively-coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>MMPs</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>FR</td>
<td>folate receptor</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>DBCO</td>
<td>dibenzylcyclooctyne</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diethylisoprylamine</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>EA</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
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Chapter 1: Introduction
1.1 Scope of Thesis

Herein is discussed progress towards detection of intracellular Ca\textsuperscript{2+} flux by magnetic resonance imaging. Two approaches will be discussed, both of which rely on Ca\textsuperscript{2+}-responsive contrast agents: \textit{in vivo} evaluation of a previously-developed esterase-activated contrast agent; and synthetic development of new Ca\textsuperscript{2+}-responsive contrast agents with a cell-transduction domain for improved cellular uptake.

Chapter 1 sets the stage for relevant background knowledge pertinent to the work in this thesis, including physics nuclear relaxation and magnetic resonance imaging, contrast agents, function and design parameters for bioresponsive contrast agents, biology of Ca\textsuperscript{2+} flux, and a perspectus of different methods for detection of intracellular Ca\textsuperscript{2+}.

The first project discussed in Chapter 2 built on the work of previous lab members Dr. Ying Song and Dr. Zhidong Ma, both of whom developed the esterase-activated agent Gd-DOPTA-Et. Evaluation of this agent in two animal models was performed in this body of work, with the goal of first determining whether the agent was capable of detecting physiologically-relevant intracellular Ca\textsuperscript{2+} flux, and then using the agent to detect increases in intracellular Ca\textsuperscript{2+} levels in a seizure model. Although it was determined that the Gd-DOPTA-Et agent was not able to report on intracellular Ca\textsuperscript{2+} \textit{in vivo}, several valuable conclusions were drawn that influenced design of the next-generation agents discussed in Chapter 3 and led to creation of an improved animal seizure model system.

The second project discussed in Chapter 3 sought to develop a new class of Ca\textsuperscript{2+}-responsive MRI contrast agents. These Gd-DOPTA-Fluor agents were designed to incorporate a cell-transduction domain which was hypothesized to increase cellular uptake of the agents and thereby
facilitate successful detection of intracellular Ca$^{2+}$ flux. Four new agents were synthesized and subjected to initial characterization. Although prone to aggregation, these new agents retain response to Ca$^{2+}$ and need to be evaluated further in biological systems.

### 1.2 Molecular imaging

The goal of molecular imaging (MI) is to elucidate biochemical events and molecular interactions non-invasively in real time. At both the clinical and pre-clinical level, MI techniques are very powerful for studying physiological pathways and progression of diseases over time.$^1$ Thus the ultimate endpoint for most MI endeavors is to shed light on pathways relevant to disease progression and treatment towards improving outcomes at the clinical level.

There are two primary factors that usually determine the choice of imaging modality used in MI: sensitivity and depth penetration. Sensitivity is straight-forward; if the target biomolecule is expressed by tissues at nanomolar (nM) concentrations, the modality used must have a nM or sub-nM detection limit. Penetration depth requirements have a little more flexibility, owing to localization of biomolecules of interest and size of the system or organism being studied. For example, a researcher studying a biochemical pathway in immortalized cell lines requires a sub-micron penetration depth, whereas studying the same pathway in mammals will most likely require a penetration depth of several centimeters.$^2$

The current MI modality most used in the pre-clinical setting is optical imaging, owing to its sub-picomolar sensitivity, ease of image acquisition, and ready availability at most research institutions.$^1,^3$ However, as one moves toward model systems with more clinical relevance (e.g. rodents), optical techniques are more challenging to implement non-invasively due to poor penetration depth of visible light through tissue.$^4$ Owing to absorption by various functional
groups in the biological milieu, the tissue penetration depth of standard fluorescent techniques is less than 1 cm, with light scattering by water and other tissue components reducing resolution at this depth into the mm range.\textsuperscript{1,5} While penetration depth can be increased to 2 cm via use of near infrared (NIR) and luminescent probes, resolution does not improve.\textsuperscript{6} This does not preclude use of optical modalities in \textit{in vivo} models that do not require high resolution detection, but places strict limits on preclinical models accessible to optical probes. Thus use of optical modalities for MI is largely limited to tissue culture and \textit{in vivo} systems made accessible to light via varied invasive methods.\textsuperscript{7} For example, craniotomy followed by placement of a cranial window allows use of fluorescent proteins and optical small molecule probes in mammalian sensory cortices.\textsuperscript{8}

In clinical and larger pre-clinical animal models, the MI modalities of choice are positron emission tomography (PET) and single photon emission computed tomography (SPECT), arising from their high sensitivity and relatively high instrument availability.\textsuperscript{1,6,9-10} Both of these methods rely on nuclear emission events, lending them picomolar sensitivities and zero background signal. However, PET and SPECT have some serious drawbacks. In addition to inherent radioactivity rendering them toxic to the subject being imaged, they also suffer from relatively low resolution (1-2 mm) and inability to detect anatomical features; instead they are specific to detection of probe emission.\textsuperscript{11} Therefore, both PET and SPECT are must be used in tandem with an anatomical imaging modality, most often X-ray computed tomography (CT).\textsuperscript{12}

Magnetic resonance imaging (MRI) occupies a unique niche in the MI paradigm, owing to its unlimited depth penetration, excellent depiction of anatomical features (including soft-tissue contrast), and lack of ionizing radiation.\textsuperscript{13} In addition, MRI has relatively high spatial resolution compared to nuclear techniques (down to tens of microns at research field strengths) and allows
for bioresponsive imaging owing to physics of probe design/function.\textsuperscript{14-15} This combination of attributes make MRI uniquely suited for longitudinal studies in intact animal models without concomitant radiation exposure.

The drawback of MRI for MI applications is the inherent low sensitivity of all MR techniques towards their probes. To date, the most sensitive MRI probes are capable of detection at mid- \( \mu \text{M} \) concentrations.\textsuperscript{16-20} As many interesting targets for MI in the proteome/metabolome are expressed at nanomolar levels, this is a huge challenge facing those trying to utilize MRI for MI. However, with appropriate target selection and savvy probe design, MRI retains potential for non-invasive MI in mammalian models.

1.3 Magnetic Resonance Theory

Magnetic resonance techniques draw their source of signal from excitation and relaxation of nuclear spins an applied magnetic field. Since different tissues provide distinct magnetic environments, they will also have intrinsic differences in the relaxation of their nuclear spins. These differences in relaxation properties are what give rise to signal in MR techniques and can be exploited to create 2D spectra in standard nuclear magnetic resonance (NMR) spectroscopy and 3D images in MRI. Although all spins with a non-zero magnetic moment can be used in MR applications, protons are the most commonly analyzed nucleus and the focus of the MR imaging in this work and thus all further discussion will be specific to \(^1\text{H}\) nuclei.

1.3.1 Behavior of Protons in an Applied Magnetic Field

In an applied magnetic field \((B_0)\), all nuclei with a non-zero spin quantum number \((I)\) will have a magnetic moment \((\mu)\) via equation 1.1,

\[
\mu = \gamma m \quad (1.1)
\]
Where $\gamma$ is the gyromagnetic ratio intrinsic to a nucleus and $m$ is the magnetic quantum number, which has $2I + 1$ energy states and takes integer values from $-I$ to $I$. This magnetic moment gives rise to excitation and relaxation of nuclear spins in all MR techniques. In the case of protons, $I = \frac{1}{2}$ and $m$ can be either $\frac{1}{2}$ or $-\frac{1}{2}$, giving rise to two distinct possible energy states populated in a Boltzmann distribution: aligned in the direction of $B_0$ and aligned in the opposite direction of $B_0$. Conventionally, the $z$-axis is oriented with $B_0$, so the net magnetization along $B_0$ (the sum of $\mu_z$) is $M_z$. It is worth noting here that $^1H$ nuclei have a 99.98% isotopic abundance and have the highest value for $\gamma$, giving rise to a very high $M_z$, from which is derived the source of signal in MR experiments. In the case of the MR imaging in this work, signal is derived from $^1H$ nuclei in water, which make up an average of 60% of biological tissues. The combination of a large $M_z$ and universal role of water in biology $^1H$ MRI ideally suited for imaging biological specimens.

As a consequence of their magnetic moment, proton nuclei in a magnetic field experience a torque.

$$\tau_z = \mu_z \times B_0 \quad (1.2)$$

This in turn forces the nuclear spins to precess about the axis of $B_0$ with a characteristic angular frequency known as the Larmor frequency ($\omega_0$), where

$$\omega_0 = \gamma B_0 \quad (1.3)$$

Thus for a constant $B_0$ along the $z$-axis, proton nuclear spins will be processing about the $z$-axis at the Larmor frequency and occupy the two permitted energy states, with a slight excess aligned with $B_0$. The difference in energy between the two populations of spins is given by

$$\Delta E = \gamma \hbar B_0 \quad (1.4)$$
where \( \hbar \) is the reduced Planck’s constant. The gap between the energy states falls into the radiofrequency (RF) range and MR spectroscopic techniques thus use RF energy to excite nuclear spins (Figure 1.1).

In the prototypical \(^1\text{H}\) MR experiment (Figure 1.2), the net magnetization begins in alignment with \( B_0 \) along the z-axis. The net \( M_x \) and \( M_y \) are both equal to zero due to precession. Upon application of an additional magnetic field \( B_1 \) at RF frequency (termed an “RF pulse”), the spins are torqued such that \( M_z = 0 \) and \( M_x \) and \( M_y \) are non-zero. The duration and direction of the RF pulse determines the flip angle (\( \alpha \)) to which the net magnetization is shifted from the z-axis (in the case of the experiment in Figure 1.2, \( \alpha = 90^\circ \)). Immediately after the removal of \( B_1 \), the spins begin to relax back to equilibrium, such that the net magnetization returns to \( M_z \). This occurs through two independent mechanisms: dissipation of RF energy back to the surroundings to regenerate spins aligned with \( B_0 \) \( (M \rightarrow M_z) \) and dephasing of spins precessing in the xy plane to return \( M_x \) and \( M_y \) to zero. The former relaxation mechanism is termed spin-lattice or “\( T_1 \)” relaxation, while the latter is termed spin-spin or “\( T_2 \)” relaxation and occurs because differences in magnetic environment (for example presence paramagnetic species) give rise to a shift in precession frequency (see Equation 1.3). \( T_1 \) is defined as the time required to regenerate 67% of \( M_z \) after a 90° RF pulse, while \( T_2 \) is defined as the time it takes for 37% dephasing of \( M_{xy} \). These relaxation mechanisms occur at characteristic rates for a given magnetic environment/tissue and are mathematically governed by the Bloch equations

\[
M_z(t) = M_{z,eq} - [M_{z,eq} - M_z(0)]e^{-t/T_1} \quad (1.5)
\]

\[
M_{xy}(t) = M_{xy}(0) e^{-t/T_2} \quad (1.6)
\]

Figure 1.1 – Behavior of $^1$H nuclear spins in an applied magnetic field. A) The magnetic moment vector of each spin precesses about the axis of $B_0$ at the Larmor frequency. B) Spins align with and against $B_0$, with a slight excess in the lower energy state. The gap between the two energy levels increases with field strength and falls within the RF-excitable range.
Figure 1.2 – MR pulse experimental overview. A) The net magnetization aligns with $B_0$ along the $z$-axis. B) Upon application of an additional magnetic field $B_1$ at RF frequency (an RF pulse), the net magnetization vector is torqued out of alignment at flip angle $\alpha$ determined by the duration of the pulse, with the result that C) net magnetization ends up in the $xy$ plane. D) During spin-lattice ($T_1$) relaxation, spins dissipate energy to their surroundings and realign with $B_0$. E) During spin-spin ($T_2$) relaxation, spins diphase in the $xy$ plane as minute differences arise in $\omega_0$ owing to field inhomogeneities. F) Both $T_1$ and $T_2$ processes occur simultaneously, with the result that spins precess back into alignment with $B_0$. 
1.3.2 MRI - from $T_1$ and $T_2$ to 3D Image

At the most basic level, an MR spectrometer functions by exciting $M$ into the xy plane and then reading out net procession of groups of spins (termed “spin packets”) in the xy plane. The spectrometer accomplishes this via receiver coils which detect magnetic oscillations in the xy plane generated by spin packets precessing at discreet Larmor frequencies. These oscillations generate corresponding eddy currents in the receiver coils of the spectrometer according to Lenz’s Law. These incredibly convoluted currents are a summation of the free induction decay (FID) of all the spin packets in the sample and can be subjected to a huge amount of computational power to Fourier transform them from the time domain to the frequency domain. Because the receiver coils only detect oscillations in the xy plane, dependent upon the sequence of RF excitation pulses used and the time(s) allowed for relaxation, the instrument can select for signal focused on various attributes of a material’s magnetic properties, including the density of spins in a region (proton density-weighting), short $T_1$ ($T_1$-weighting), and short $T_2$ ($T_2$-weighting), in addition to many others. For an excellent coverage of signal generation and pulse sequences in conventional MRI, see reference 21.

In order to deconvolute a meaningful 3D image from the summation of eddy currents generated in a sample, each discreet spatial location being imaged (termed a “voxel”) must be encoded with a unique frequency tag for image reconstruction post-Fourier transformation of the summed FID. MRI scanners accomplish this via imposition of discreet ramped field gradients in $B_0$ along both the x and y axes. Thus for each (x,y) coordinate, spins will have a unique Larmor frequency and a 3D image can be constructed from compiling many 2D xy slice images. This is the basis for formation of 3D tomographic images in all MR imaging techniques.
Differences in signal intensity between voxels is a function of the pulse sequence used and the magnetic properties of the voxel contents, and gives rise to image contrast in the MR images generated. For example, in $T_1$-weighted images, voxels with shorter $T_1$ appear bright (positive contrast) and in $T_2$-weighted images, voxels with shorter $T_2$ appear dark (negative contrast). Intrinsic magnetic differences between tissue types are able to generate anatomical MR images without introducing exogenous probes. However, this same high native contrast in MR images also gives rise to large amounts of background signal that is independent of the MR probe under investigation. In combination to poor probe sensitivity (see Section 1.4), high background signal further challenges researchers attempting to use MRI for MI applications.

For most biological applications, MR pulse sequences and/or probes that generate positive image contrast are preferable to those that generate negative contrast, because very few native biological components/processes generate positive contrast, while a lot of native biomaterials generate negative contrast (for example air pockets, regions without water such as fat and bone, etc.).

### 1.3.3 Functional MRI

Functional MRI (fMRI) is broadly defined in the literature as MRI images that measure a physiological response or activity. However, the term is almost always used in association with generation of blood oxygen-level-depend (BOLD) contrast used to depict regions of neuronal activity in the central nervous system (CNS).\(^{22}\) fMRI is responsible for much of what is currently known about the function of regions in the clinical setting and has led to huge amounts of data in deep-brain regions inaccessible to optical agents in preclinical animal models. BOLD contrast generation stems from the haemodynamic response phenomenon, whereby regions undergoing
neuronal activity receive more oxygenated blood than inactive regions. The haemodynamic response is generated by astrocytes, pericytes, and the smooth muscle composing vascular walls and results in a net dilation of capillaries supplying active neurons. Since oxygenated blood has a higher ratio of oxy- to deoxy-hemoglobin; and since oxy-hemoglobin contains paramagnetic Fe(III), regions with more oxygenated blood will be susceptible to slight changes in $T_2$.

Typical fMRI experiments utilize pulse sequences that are heavily $T_2$- or $T_2^*$- weighted to highlight the minute field inhomogeneities generated by Fe(III) in oxy-hemoglobin ($T_2^*$ takes into account field inhomogeneities arising from an imperfect $B_0$ and is typically much faster than $T_2$; $T_2^*$-weighting is best for generation of image contrast from point defects in $B_0$ which include Fe(III)). However, these differences in $T_2/T_2^*$ are very minute and typically fall within intrinsic noise of the scanner (e.g. inhomogeneity in $B_0$). BOLD contrast therefore typically requires multiple stimulation/readout steps and always necessitates a huge amount of post-acquisitional image processing. Once BOLD contrast has been generated, it is shown as an overlay on the anatomical MR image.

Despite the huge amount of knowledge gained from fMRI experiments, there is significant room for improvement, stemming from uncertainties in the haemodynamic response phenomenon and erroneous findings arising from complications in post-acquisitional image processing. First, while there is a known correlation between increased oxygenation and neuronal activity, the haemodynamic response is still not fully understood. Additionally, maximal BOLD signal arises several seconds after neuronal firing, hampering temporal resolution of fMRI and constraining spatial resolution by minimizing acquisition times. The most significant drawback to fMRI however, is requirement of extensive processing required to tease out regions with positive BOLD
contrast. This requires several algorithms that distort the dataset and result in both poor resolution and can yield false data. For example, researchers have been able to generate BOLD contrast in the brains of dead specimens using standard processing algorithms.\textsuperscript{28}

1.4 Contrast Agents

Although MRI is able to generate anatomical images based on native differences in the magnetic properties of different tissue types, it is not necessarily able to depict differences in histology that are prerequisite to MI applications. To put it simply, unique histology does not imply unique magnetism. In order to address this issue, MR probes can be used to change the magnetic environment of water protons with which they interact. These probes are termed “contrast agents” (CAs), as they enhance MR image contrast where they are localized. It is because MR techniques detect the effect of the probe (e.g. relaxation enhancement) rather than directly detecting the probe itself, that gives rise to low sensitivity compared to other MI techniques.\textsuperscript{1}

MR CAs fall into two categories: $T_1$ agents (which decrease $T_1$ and generate positive image contrast) and $T_2$ agents (which decrease $T_2$ and generate negative image contrast). However, this is largely semantic since paramagnetic species affect both $T_1$ and $T_2$. Usually the category an agent falls into is actually a consequence of MR image acquisition. As discussed previously, MR spectrometers only detect in the xy plane. If dephasing of spin packets in a region occurs faster than the instrument can refocus the spins and receive frequency information (e.g. an area of very short $T_2$), no $T_1$-weighted signal will be generated, a process known as “$T_2$ quenching”.\textsuperscript{21} Thus, classification of a CA as a $T_1$ or $T_2$ agent is usually based on whether it can decrease $T_1$ by a measurable amount without concomitant $T_2$ quenching. In addition, $T_1$ agents can be utilized as $T_2$ probes by simply changing the pulse sequence used for imaging. This work has exclusively
used small-molecule $T_1$ CAs containing Gd(III) and thus all further discussion will pertain to probes of this nature.

1.4.1 Gd(III) Contrast Agent Theory

All paramagnetic species are able to modulate $T_1$/$T_2$ of water protons to some degree. However, their efficacy is by no means equal. Most $T_1$ CAs utilize Gd(III) because it does this better than other ions and because it forms stable complexes under biological conditions. The latter is important, because free Gd$^{3+}$ provides few opportunities for MI and is renally toxicity. Thus, current clinically-approved Gd(III) CAs utilize either macrocyclic ligands or linear ligands with many chelating groups for stable coordinate of Gd(III), although macrocyclic ligands are preferred owing to higher stability (Figure 1.3).

In the ground state, Gd(III) has 7 unpaired f-electrons, giving it a very large magnetic moment and facilitating $T_2$ relaxation of water protons. What makes Gd(III) unique among other very highly paramagnetic lanthanide species such as Dy(III) and Ho(III), is that Gd(III) has a symmetric S-state, which gives it an electronic relaxation time ($T_{1e}$) several orders of magnitude slower than its asymmetric counterparts ($\sim 10^{-7}$ s). This is critical for success as a $T_1$ CA, because for an optimized CA,

$$\frac{1}{\tau_c} = \frac{1}{\tau_R} + \frac{1}{\tau_m} + \frac{1}{T_{1e}} \approx \omega_0 \quad (1.7)$$

Where $\tau_c$ is the correlation time, $\tau_R$ is the rotational correlation time, $\tau_m$ is the mean residence lifetime of a bound water molecule ($1/k_{ex}$), and $\omega_0$ is the Larmor frequency, as described by Solomon-Bloembergen-Morgan (SBM) theory. At research field strengths (>1.5 T), $\omega_0$ is
Figure 1.3 – Structures of some Gd(III) CAs commonly used in the clinic. The upper row contains macrocyclic chelates, while the lower row contains linear chelates. All clinical agents are at least 8-coordinate with respect to Gd(III) to maintain stable chelation.
on the order of $10^{-9}$ s and $\tau_R$ and $\tau_m$ typically fall within an order of magnitude of this value. Thus the $T_{1e}$ of Gd(III) is not fast enough to dominate $1/\tau_c$ and deviate it from optimal. The primary mechanisms for the interaction of Gd(III) with water protons are the dipole-dipole and scalar coupling interactions between the proton nuclear spins and the oscillating magnetic fields surrounding Gd(III) as a result of its favorable $T_{1e}$.\textsuperscript{6,15,31}

The ability of a Gd(III) CA to enhance $T_1$ relaxation is termed its “relaxivity” ($r_1$; in units of mM\textsuperscript{-1}s\textsuperscript{-1}). Thus for a given sample, the measured $T_1$ ($T_{1,obs}$) is given by

$$\frac{1}{T_{1,obs}} = \frac{1}{T_{1,d}} + r_1 [Gd(III)] \quad (1.8)$$

Where $T_{1,d}$ is the diamagnetic contribution to $T_1$ (e.g. the native $T_1$ in the absence of any CA). The dominant mechanisms of relaxation by Gd(III) CAs occurs through dipole-dipole coupling.\textsuperscript{33} Hence the ability of these agents to interact with water protons falls off rapidly with distance ($1/r^6$), such that there are several hydration shells that contribute to relaxation (inner-sphere, second-sphere, and outer-sphere; see Figure 1.5).\textsuperscript{32}

1.4.2 Inner-sphere Relaxion

The inner-sphere (IS) of water molecules is defined as those directly coordinated to the Gd(III) center. As the closest \textsuperscript{1}H nuclei to the paramagnetic center, they are responsible for the greatest contribution to relaxivity. Several factors affect IS relaxation, including $q$ (the number of water molecules coordinated to Gd(III)), $\tau_R$, and $\tau_m$ (Figure 1.4). These attributes are readily modulated by chemical modifications to chelate structure and will hence be the focus of this discussion.
Figure 1.4 – Parameters that govern IS water relaxation by Gd(III) CAs.
The paramagnetic contribution to inner sphere relaxation ($T_{1,p}$) is governed by

$$\left(\frac{1}{T_{1,p}}\right)^{\text{IS}} = \frac{c q}{55.5} \left(\frac{1}{\tau_{1m} + \tau_m}\right) = \rho_m q \left(\frac{1}{\tau_{1m} + \tau_m}\right) \quad (1.9)$$

Where $c$ is the molality of the CA, $T_{1m}$ is the longitudinal relaxation time of the coordinated water molecule, and $\rho_m$ is the mole fraction of coordinated water. As evident from equation 1.9, the contribution from $q$ is linear; however clinically relevant Gd(III) CAs have $q \leq 2$, owing to a decrease in thermal stability of the chelate at higher $q$ values. For a complete discussion of contributions to inner-sphere relaxivity, see references 14 and 32.

Qualitatively, a CA must be optimized such that it interacts with enough water molecules to effectively transmit relaxation to the bulk water. For IS relaxation mechanisms, $q$ is the most straight forward: if the chelate does has no open coordination site(s) for water to bind, then it will not relax water protons via IS mechanisms. $\tau_m$ is optimized when a bound water resides on the Gd(III) center just long enough to interact (10-50 ns at research field strengths). Most small-molecule Gd(III) CAs suffer from $\tau_m$ values that are at least an order of magnitude to slow. The typical method of decreasing $\tau_m$ into the optimal range is to increase steric bulk of the ligand around the water-binding site by synthetic methods. $\tau_R$ is optimized when tumbling of the CA through the bulk water is slowed down such that it is on the same order of magnitude as the exchange rate (1-10 ns at research field strengths). Typically, this is accomplished by increasing the effective molecular weight of the CA, either by tethering the agent to a larger scaffold or decreasing the rotational degrees of freedom between the Gd(III) chelate and the rest of the covalent complex. It is worth noting here that both $\tau_m$ and $\tau_R$ are field-dependent, so CAs must be optimized for the field strength at which they will be used. However, the general trend holds that decreasing $\tau_m$ and increasing $\tau_R$ will maximize agent performance.
1.4.3 Second and Outer Sphere relaxation

The second sphere (SS) of water molecules interacting with a CA is defined as those water molecules that interact with the chelate but do not directly coordinate to the Gd(III) center. These interactions include hydrogen bonds and various other dipole-dipole interactions. The outer-sphere (OS) comprises the water molecules that still interact with the CA while diffusing by it, but are not in the IS or SS (Figure 1.5). While SS and OS relaxation mechanisms are less well-understood than their IS counterparts, they still make a significant contribution to $r_1$ (up to 40%). In addition, SS and OS effects can dramatically increase $r_1$ in macromolecular systems with a high degree of ordered water, such as nanoparticles. For an in-depth review of Gd(III)-based nanoparticle CAs, see reference 38.

It is not straightforward to directly measure SS and OS contributions to relaxation due to ambiguity in the number of waters comprising these shells and the short-lived nature of their interaction with the CA. It is very tricky to empirically determine the number of water molecules hydrogen bonded to a chelate, let alone to determine the number of bulk waters passing by the CA at any one point in time. However, there are equations that give an approximation of SS and OS contributions by simplifying the CA and the water molecules to hard spheres and calculating the number of interactions they have based on CA size, concentration, diffusion, etc. Another more empirical method is to measure $r_1$ of a CA and subtract $r_1$ of a coordinatively saturated analog to determine IS and SS/OS contributions to overall $r_1$. This presents its own obvious challenges. Usually, nuclear magnetic relaxation dispersion (NMRD) analysis and curve fitting, often combined with electron paramagnetic resonance (EPR) and $^{17}$O NMR studies are required to more-
Figure 1.5 – Hydration shells of Gd(III) CAs. IS water(s) directly coordinate the metal center and modulate CA relaxivity via $q$, $\tau_R$, and $\tau_M$. The SS is largely comprised of water molecules hydrogen bonded to the chelate, while the OS is made up by molecules that are spatially close enough to interact with the Gd(III) while diffusing past the CA, but are not in the SS.
fully tease out more subtle aspects of SS/OS contributions to $r_1$ by fully solving for parameters governing for IS relaxation.$^{14-15,32,41}$

Of import to this work are two consequences of SS and OS contributions to $r_1$. The first is that Gd(III) CAs with coordinatively saturating ligands ($q = 0$) maintain 30-40% of their relaxivity owing to SS and OS effects; thus bioactivatable agents that have a turn-on response from increasing $q$ have a very significant background signal. The second point is that supramolecular CAs such as nanoparticles can have much higher relaxivities than their small molecule counterparts due to enhanced SS and OS contributions.

1.4.4 Relaxivity Versus Gd(III) Payload - the Nanoparticle Strategy

Revisiting equation 1.8, it is clear that there are two non-mutually exclusive approaches for making a better CA: increasing the relaxivity of the CA and increasing its concentration at the region of interest (the Gd(III) “payload”).

$$\frac{1}{T_{1,obs}} = \frac{1}{T_{1,d}} + r_1[Gd(III)] \quad (1.8)$$

The parameters governing CA performance that are readily modified through chemical methods ($q, \tau_R, and \tau_m$) have already been discussed and strategies for modulating these properties to yield bioresponsive CAs for MI applications will be discussed in the next section.

The most common method of increasing Gd(III) payload delivered to the region of interest is to tether Gd(III) chelates onto a nanoplatform of choice, whether it be organic polymer-based or inorganic.$^{38}$ While this strategy is often synthetically straightforward, it has several significant caveats affecting $r_1$ of the nanoconjugate CA and its behavior in biological systems. Although some nanoplatforms can achieve a huge boost in $r_1$ over their small molecule counterparts due to SS and OS effects from a more ordered hydration shell, others are not so blessed. In the absence
of SS/OS contributions, many nanoconjugate CAs have a disappointing $r_1$/Gd(III) (the “ionic $r_1$”) as a function of linker chemistry to the nanoplatform owing to increases in $\tau_R$ if the Gd(III) chelate tumbling is uncoupled from the tumbling of the nanoconjugate.\textsuperscript{42} Dendrimeric CAs often fall into this category and as a result are often not as promising for MI applications as more rigid nanoconjugates such as those utilizing silica, gold, or carbon at their base core.\textsuperscript{43-46}

Although a low ionic $r_1$ can be overcome by increasing the number of Gd(III) chelates that contribute to the overall $r_1$ of the nanoconstruct (the “molecular $r_1$”), the change from small molecule to nanoplatform has an irreversible effect on CA pharmacokinetics. While small molecule CAs often exhibit rapid renal clearance, they are also capable of diffusing freely into tissue and escaping capillary networks, not to mention crossing biological membranes and accessing cytosolic contents.\textsuperscript{47} Nanoconjugates undergo different clearance mechanisms and have poor perfusion through tissues owing to their larger size.\textsuperscript{48-50}

The most significant caveat of nanoconjugate CAs for MI applications, however, is that they do not have direct access to cytosolic components. It is generally accepted that nanoparticles enter cells via interaction with scavenger receptors followed by endocytosis.\textsuperscript{51-52} Thus, to bind intracellular targets for MI application, nanoparticles must undergo so-called “endosomal escape.” There is currently much debate over how this occurs, but a common theme is that in order to be successful, the nanoconjugate must release a small molecule payload into the endosome, from which the released small molecules have the potential to escape.\textsuperscript{53-55} The best way for nanoconjugate CAs to avoid failure due to encapsulation in endosomes is simply to have targets that are expressed on the outer surface of cells. Since many biomolecules involved in signaling
and cellular response are surface exposed, this retains a large pool of potential targets for MI applications.

The last aspect of nanoconjugate CAs that must be overcome for MI applications is high nonspecific uptake of nanoparticles. Endocytosis of nanoconjugates does not rely on binding of the nanoparticle to its targeted receptor. Therefore in order for a nanoconjugate to generate detectable image contrast at the target tissue, it must not only penetrate to and accumulate at that tissue in amounts above the detection limit of MRI, but achieve this at levels above the background of nonspecific probe uptake. This affectively raises the detection limit of nanoconjugate MR probes to the standard MR detection limit plus the background. Despite the challenges facing use of nanoconjugate CAs for MI, with appropriate target selection and choice of in vivo model and experimental protocol, there are a few successful Gd(III) nanoprobes in the literature.

1.5 Bioresponsive Gd(III) CAs

Most MI endeavors belong to one of two categories: reporting location and expression level of biomarkers or relating biological processes such as enzyme activity. The first largely falls under the purview of targeted probes: chemically modify a targeting moiety with an imaging agent. Although this presents a host of challenges for targeted MR probes due to the inherent high detection limit and background signal, it is a strategy that usually is relatively straightforward and will not be discussed here. For excellent reviews, see the following. The rest of this section will focus on bioactivatable MR probes that generate a change in MR signal upon interaction with a target biomarker or biological event. These probes achieve this by modulation of their $r_1$ through a corresponding change in the parameters that affect IS relaxation: $q$, and $\tau_R$ ($\tau_m$-modulation for
MRI has not been successful to-date; modulation of water exchange typically falls under the purview of paraCEST imaging - for an excellent review see reference 62). 6, 18-19, 60, 62-64

1.5.1 $\tau_R$-Modulated CAs

Activatable MR CAs that are $\tau_R$-modulated generate an increase in $r_1$ by slowing molecular tumbling. Typically, this occurs via interaction of the CA with a biomacromolecule or by inducing aggregation of the CA in response to a biological stimulus (Figure 1.6). The vast majority of the former class CAs are targeted probes that generate contrast at the target site through accumulation of Gd(III) and a bump in relaxivity of target-bound probe without changing the structure of the probe. Well-designed targeted MR probes are designed with a $\tau_R$-boost in mind and can thus be said to be $\tau_R$-modulated.

There are not as many $\tau_R$-modulated bioactivatable/bioresponsive MR probes, owing to the fact that most chemical reactions that will change the chemical structure of the CA lead to cleavage products that are smaller and tumble faster than the original probe. However, there are many examples of successful “turn-on” (i.e. higher $r_1$ in the activated state) $\tau_R$-modulated CAs in the literature. 65-69 One of, if not the slickest representative approaches to bioresponsive $\tau_R$-modification has been investigated in the Rao Lab to image localization and activity of apoptotic caspases in vivo. 70 The authors incorporated two bioresponsive domains that, when activated, lead to probe aggregation and a large increase in $\tau_R$. The first bioresponsive element is a caspase-cleavable peptide sequence which unMASKs an amino group. The second is a disulfide-protected thiol group unmasked by the reducing intracellular environment. Only when the probe has traversed the plasma membrane in caspase-expressing cells will both the amine and the thiol be available to
Figure 1.6 – $\tau_R$-modulation strategies. A) Binding of a small molecule probe to a biomacromolecule will increase $\tau_R$. B) Probe aggregation in response to a biological stimulus (pH, enzyme activity, etc.) can yield a large change in effective molecular weight and provide a $\tau_R$-modulated boost in relaxivity.
carry out an intramolecular cyclization reaction which leads to probe self-assembly and a turn on response in probe contrast enhancement via $\tau_R$ (Figure 1.7).

1.5.2 $q$-Modulated CAs

The other parameter governing IS relaxivity that is readily accessible to biological activation is $q$. $q$-modulation is an attractive strategy for bioresponsive MR probes, because unlike contributions to IS $r_1$ from $\tau_R$, $q$ is field-independent. Thus, there is no need to optimize a $q$-modulated CA for a particular magnetic field strength. In addition, $q$-modulation is dependent solely on the coordination environment of Gd(III) and therefore generates predictable $r_1$ changes.

$q$-modulated CAs are activated via dissociation of a bioresponsive domain from the Gd(III) center. This frees up a coordination site for IS water molecules and leads to an increase in $r_1$. There are three primary ways to release the bioresponsive domain from coordination to Gd(III): enzymatic cleavage, preferential coordination to an analyte (most often an ion), and chemical reaction (usually redox-based) of the bioresponsive domain with its analyte such that it no longer coordinates Gd(III) (Figure 1.8). The Meade Lab has pioneered the first two areas, developing the first bioresponsive cleavable CA (EGad, which detects $\beta$-galactosidase activity) in 1997 and the first ion-responsive CA (Gd-DOPTA, which detects $Ca^{2+}$) in 1999. To date $q$-modulated Gd(III) CAs responsive to pH, $Ca^{2+}$, $Zn^{2+}$, $K^+$, $Cu^{1+/2+}$, $Fe^{2+}$, and a host of enzymes and reactive redox species have been developed.

1.6 Molecular Imaging Methods for Detecting $Ca^{2+}$ Flux in the CNS

The calcium ion is the primordial intracellular signaling analyte and is ubiquitous in all biological systems. Gradients and fluctuations in intracellular $Ca^{2+}$ concentration play critical roles in nearly all cellular events, including membrane potential maintenance, action potential
Figure 1.7 – \( \tau_R \)-modulation approach taken by Rao and coworkers. Pre-activation by cytosolic reductants and caspase, the probe is monomeric with a fast \( \tau_R \). Once the amino and thiol groups are unmasked, an intracellular cyclization reaction occurs. The cyclic product aggregates and yields a large \( \tau_R \)-modulated \( \Delta r_1 \) boost.
Figure 1.8 – q-modulation strategies. The bioresponsive domain initially blocks water access to the Gd(III) center, resulting in a low $r_1$ “off” state. In response to A) analyte-binding, B) enzymatic cleavage, and C) chemical modification, the bioresponsive domain no longer coordinates, freeing up an IS water coordination site and leading to a high $r_1$ “on” state.
propagation, signal transduction cascades, and apoptosis. In vertebrates, Ca\(^{2+}\) is the fundamental transducer of electrical activity in the CNS, and supports all aspects of electrical signaling. Although CNS action potentials are Na\(^{+}\)-dependent, influx of Ca\(^{2+}\) into the cytosol is not only responsible for action potential initiation and propagation, but also instigates interneuronal communication via release of neurotransmitters at the synaptic gap to transmit the electrical signal to adjacent neurons. Aside from short-term signaling events, Ca\(^{2+}\) also activates gene expression via instigation of signal transduction cascades that convert external stimuli to changes in the proteome (Figure 1.9). In order to achieve rapid depolarization in response to opening of Ca\(^{2+}\) channels, neurons maintain a very high concentration of extracellular Ca\(^{2+}\) (2-5 mM) and very low resting-state levels of intracellular Ca\(^{2+}\) (low-mid nM). Thus, while intracellular [Ca\(^{2+}\)] may change by many orders of magnitude during signaling events, the extracellular Ca\(^{2+}\) “pool” remains relatively constant in comparison.

Since intracellular Ca\(^{2+}\) flux is critical to proper neuronal function, dysregulation of Ca\(^{2+}\) flux has been implicated in a host of CNS disorders, including autism, schizophrenia, and epilepsy. Abnormal glutamatergic signaling is often linked to Ca\(^{2+}\) dysregulation, because glutamate receptors also function as Ca\(^{2+}\) channels. In some cases, glutamatergic hyperfunction gives rise to excitotoxicity, which involves repetitive influx of large amounts of Ca\(^{2+}\) into the cytosol that can result in tissue damage as neurons deplete cellular energy reserves in attempt to efflux the Ca\(^{2+}\). Excitotoxicity a root cause of neuronal damage arising from many seizure disorders. The importance of Ca\(^{2+}\) flux in neuronal signaling has led to the development of Ca\(^{2+}\)-responsive probes for use in MI, including optical and MR agents.
Figure 1.9 – Neuronal Ca\(^{2+}\) signaling pathways. Neurons maintain a huge Ca\(^{2+}\) concentration gradient across the cell membrane for rapid responses to opening of voltage- and ligand-gated ion channels. The red asterisks indicate ligand-gated Ca\(^{2+}\) channels which are involved in glutamate signaling. Adapted from reference 77.
1.6.1 Optical Ca\(^{2+}\) Sensors

All small-molecule Ca\(^{2+}\) probes are based on the structure of the chelator EGTA, which provides a binding pocket suitably sized for Ca\(^{2+}\) and other fourth row divalent cations. Important for their use in biological systems, EGTA-based Ca\(^{2+}\) sensors are not able to accommodate Mg\(^{2+}\) well. Thus, they can be said to be Ca\(^{2+}\)-selective due to the scarcity of non-protein bound transition metals in the biological milieu. The first Ca\(^{2+}\)-sensitive dye designed for use in biological systems, and the progenitor of all modern optical Ca\(^{2+}\) sensors, was BAPTA (Figure 1.10). BAPTA is able to accurately depict Ca\(^{2+}\) concentration via a change in UV absorbance upon binding Ca\(^{2+}\), stemming from a decrease in electron density in the aromatic rings upon Ca\(^{2+}\) chelation. However, BAPTA has three fundamental flaws that have prevented its use in cellular models and \textit{in vivo}. The first is that it absorbs light in the UV (\(\lambda_{\text{no calcium}} = 254 \text{ nm}; \lambda_{\text{calcium}} = 203 \text{ nm}\)), such that its signal is swallowed by absorbance of proteins and other biomolecules. The second and third flaws of BAPTA are intertwined: it is not very membrane permeable with four ionizable carboxylates and it is not blind to the millimolar extracellular Ca\(^{2+}\) pool (see Figure 1.9). Thus, even if a UV-Vis spectrometer could separate BAPTA’s signal from the biological background, it would not be reporting on the physiologically relevant intracellular Ca\(^{2+}\) level.

Synthetic structural modifications to the BAPTA core have allowed optical Ca\(^{2+}\) sensors to be employed ubiquitously in neuroscience MI endeavors. Expanding the conjugated aromatic region of BAPTA has yielded fluorescent probes which do not absorb/emit in the UV, including agents that allow precise ratiometric detection via emission shifting. Low cell permeability and sensitivity to extracellular Ca\(^{2+}\) have been solved via incorporation of enzymatically labile acetoxyethyl (AM) esters onto the BAPTA iminoacetates (see Figure 1.10). Esterification is a common prodrug strategy that raises the lipophilicity of the agent and renders the carboxylates unable to bind Ca\(^{2+}\), requiring cleavage by intracellular nonspecific esterases for release of active agent, which is unable to diffuse back across the plasma membrane, and effectively trapped in the cytosol. Although AM esters are notoriously prone to
Figure 1.10 – Commonly used chemical Ca\textsuperscript{2+} indicators. All are based on the structure of BAPTA (A). Modern indicators suitable for \textit{in vivo} use have expanded aromatic regions for longer wavelength fluorescent detection and incorporate AM esters for cell penetration and detection of intracellular Ca\textsuperscript{2+}. B) Calcium Crimson\textsuperscript{TM} is representative of single wavelength emission dyes which increase fluorescence intensity upon Ca\textsuperscript{2+} binding. C) Calcium Indo-1 is representative of ratiometric dyes which shift emission upon Ca\textsuperscript{2+} binding.
non-enzymatic hydrolysis, optical modalities can detect nM probe concentrations, with the result that the common strategy for using AM Ca\textsuperscript{2+} sensors is simply to allow extracellular/hydrolyzed dye to diffuse away prior to imaging.\textsuperscript{79, 91}

Fluorescent AM ester BAPTA analogs are still widely used by neuroscientists to detect Ca\textsuperscript{2+} both \textit{in vitro} and \textit{in vivo}, owing largely to their commercial availability in a broad spectrum of Ca\textsuperscript{2+} affinities and photophysical properties. However, genetically encoded Ca\textsuperscript{2+} sensors have become the prevalent method for optical monitoring of Ca\textsuperscript{2+} flux \textit{in vivo}.\textsuperscript{92-94} Most genetically encoded Ca\textsuperscript{2+} sensors utilize a fluorescent protein bearing a C-terminal calmodulin domain (which selectively binds Ca\textsuperscript{2+}) and an N-terminal M13 fragment (from myosin light chain kinase; a target of calmodulin).\textsuperscript{95} When the calmodulin domain binds Ca\textsuperscript{2+}, it can then interact with M13 and change conformation of the protein, with the end result being an order of magnitude increase in fluorescence. Protein-based sensors have several advantages over their small-molecule counterparts, with the most important for \textit{in vivo} applications being longer cytosolic half-lives and constitutive expression. The protein sequence can be readily introduced into neurons in a region via viral transfection post stereotaxic injection. Once the tissue is allowed to heal and stably express the protein construct, the animal can be repetitively imaged in longitudinal studies \textit{without} need for introduction of additional Ca\textsuperscript{2+} sensor.\textsuperscript{96}

Although optical Ca\textsuperscript{2+} sensors remain a key tool for neuroscience MI applications, the poor penetration depth of light in biological systems precludes their use in intact animal models. In mammalian models, optical agents require implantation of cranial windows and regions accessible to them are limited to the sensory cortices on the brain’s surface. Their use in these regions has been hugely informative, revealing signaling pathways with acute spatiotemporal resolution, but there is room for improvement. With this in mind, the Meade Lab and other research groups have sought to develop MR Ca\textsuperscript{2+} sensors.
1.6.2 Ca$^{2+}$-Responsive Gd(III) CAs

The Meade Lab developed the first Ca$^{2+}$-responsive MRI CA, Gd-DOPTA, by conjugating two Gd(III) chelates to BAPTA via a three-carbon linker (Figure 1.11). Gd-DOPTA undergoes a $q$-modulated increase in $r_1$ of 80% upon binding Ca$^{2+}$. Because it utilizes BAPTA for Ca$^{2+}$ chelation, it retains the selectivity displayed by optical Ca$^{2+}$ sensors in biological systems, although this is not as important for MR studies, since Ca$^{2+}$ is the only biological divalent cation present in concentrations above the MR detection limit. Gd-DOPTA also retains a relatively high Ca$^{2+}$ affinity ($K_d \approx 1 \mu$M), making it suitable for intracellular Ca$^{2+}$ detection in the appropriate model system (neurons can achieve 1µM intracellular [Ca$^{2+}$] during seizure events). In a proof-of-principle imaging experiment, unfertilized *xenopus laevis* eggs were injected with Gd-DOPTA and imaged before/after fertilization. Gd-DOPTA successfully enhanced MR contrast to depict increased cytosolic Ca$^{2+}$/Zn$^{2+}$ that egg cells release in response to sperm antigens during fertilization (Figure 1.12).

Gd-DOPTA has a large $\Delta r_1$ and a Ca$^{2+}$-affinity suitable for monitoring intracellular Ca$^{2+}$ in vivo, but it suffers from low cell-permeability as a result of the charges on the BAPTA domain. In addition, as with non-esterified optical Ca$^{2+}$ dyes, Gd-DOPTA is not blind to the extracellular Ca$^{2+}$ pool, such that any $\Delta r_1$ from intracellular Ca$^{2+}$ flux will be hidden by saturated extracellular probe. Thus in vivo MR images of Gd-DOPTA stereotaxically injected into the mouse CNS show a region of bright image contrast corresponding to on-state CA (Figure 1.13). To address this issue, the Meade Lab followed the esterification prodrug strategy to create the Gd-DOPTA-Et agent, which incorporates ethyl esters on the BAPTA domain. Esterification makes the agent an order of magnitude more lipophilic than Gd-DOPTA and renders it unable to detect Ca$^{2+}$ prior to cleavage.
Figure 1.11 – A) Gd-DOPTA undergoes a change in coordination upon Ca\(^{2+}\) binding which results in a corresponding 80% q-modulated increase in \(r_1\). B) The measured Ca\(^{2+}\) affinity of Gd-DOPTA is approximately 1 µM. C) MR solution phantoms of Gd-DOPTA with varying amounts of Ca\(^{2+}\).
Figure 1.1 – A) Gd-DOPTA successfully depicted changes in intracellular Ca$^{2+}$ and Zn$^{2+}$ in fertilized X. laevis eggs. The control egg received no injection of CA. False-color overlay on the eggs represents fitted $T_1$ values in those regions. Each set of 3 eggs represents a separate xy slice image.

Figure 1.12 – Coronal MR slice of a mouse brain stereotaxically injected with Gd-DOPTA. The agent is saturated by extracellular Ca$^{2+}$ resulting in positive contrast enhancement in the MR image. Image obtained at 9.4T (GRE pulse sequence; 100 micron isotropic resolution).
by intracellular esterases (Figure 1.14). Importantly, the ethyl esters are still able to coordinate to the Gd(III) centers almost as well as the unmasked carboxylates, maintaining a low $r_{1,off}$ state.

Cellular uptake and toxicity of Gd-DOPTA-Et were performed in immortalized mouse hippocampal HT-22 cells and upheld that esterification renders the agent lipophilic enough to enter cells much more readily. Gd-DOPTA-Et enters cells fivefold better than Gd-DOPTA, achieving dosage-dependent cellular loading of up to 8 femtomes/cell and yielding visible contrast enhancement in MR images of pelleted HT-22 cells (Figure 1.15). As a point of reference, a CA will most likely be detectable in MR images of pelleted cells at concentrations $\geq 0.1$ fmol/cell and detectable in vivo at concentrations $\geq 1$ fmol/cell. Thus, esterification gives Gd-DOPTA-Et the potential to reach appropriate intracellular concentrations for in vivo applications.

Although synthetic attempts were made to incorporate AM esters in place of the less biologically-hydrolyzable ethyl esters, the AM-esterified CA was too labile to be successfully isolated. However, in vitro studies with model esterases showed successful cleavage of the ethyl esters to release Gd-DOPTA.

Synthesis of Gd-DOPTA-Et follows that of Gd-DOPTA, with the exception of incorporating ethyl esters on the BAPTA core for selective deprotection of tert-butyl esters on the rest of the molecule (Scheme 1.1). Briefly, the agent is synthesized in seven linear steps beginning with 2-nitroresorcinol. In the first step, 2-nitroresorcinol is monoalkylated with 3-bromopropanol in a 34% yield, followed by dimerization with 1,2-dibromoethane in 71% yield. Next, the nitro substituents are reduced quantitatively by catalytic hydrogenolysis to anilino groups, which are subsequently di-alkylated with ethyl bromoacetate in 94% yield. The hydroxyl groups are brominated using elemental bromine in 54% yield, followed by alkylation with tert-butyl protected
Figure 1.14 – Activation of Gd-DOPTA-Et. The agent must traverse the plasma membrane and be cleaved by intracellular esterases to release active Gd-DOPTA, which can then bind Ca$^{2+}$ and report on cytosolic Ca$^{2+}$ flux.

Scheme 1.1 – Synthesis of Gd-DOPTA-Et.
Figure 1.1 – A) $T_I$-weighted MR image and corresponding color map of HT-22 cells incubated with 1, 2, and 3 and media alone for 4 h (scale bar = 1 mm). B) Calculated values corresponding to the MR image in (A). Values represent the average of four 1 mm slices and the error is plus or minus one standard deviation of the mean.
macrocycle in 65% yield. The final complex is obtained after deprotection of the acetate groups on the macrocycles in formic acid, followed by metallation with GdCl$_3$ and reverse phase HPLC purification (44% yield over the last two steps; ~3% overall yield). The use of Gd-DOPTA-Et in this thesis focuses on its evaluation *in vivo* and will discussed in Chapter 2.

In parallel to intracellular Ca$^{2+}$-responsive pursued in the Meade Lab, the Logothetis, Angelovski, and Tóth research groups have pursued *q*-modulated CAs with decreased Ca$^{2+}$ affinity, designed to monitor decreases in the *extracellular* Ca$^{2+}$ pool following neuronal activity.$^{101-104}$ These CAs use a combination strategy of decrease in the number of chelating groups in the Ca$^{2+}$-binding domain and/or utilization of EGTA instead of BAPTA to weaken Ca$^{2+}$ binding (Figure 1.16).$^{105}$ Although these CAs retain selectivity for Ca$^{2+}$ and Zn$^{2+}$, they have a much more modest $\Delta r_1$ in the presence of Ca$^{2+}$ and several are not fully *q*-modulated, instead reflecting very little change in the coordination environment of Gd(III) and owing their slight $r_1$ increase to changes in rigidity upon Ca$^{2+}$ chelation. Not surprisingly, the probe with the largest and fully *q*-modulated $\Delta r_1$ (Figure 1.16C) employs a modified Zn$^{2+}$-binding domain and is much more sensitive for Mg$^{2+}$/Zn$^{2+}$ than for Ca$^{2+}$.$^{102}$ This probe also has a $K_d \cong 10 \mu M$, rendering it too sensitive for extracellular use, but not sensitive enough to detect intracellular Ca$^{2+}$.

Logothetis and coworkers have been able to correlate transient decreases in extracellular Ca$^{2+}$ after electrical stimulation to increase in $T_1$ utilizing one of their CAs (Figure 1.16B) in cultured neurons.$^{106}$ However, there are some severe limitations to their approach. The most significant challenge for MR Ca$^{2+}$ imaging is that there is only a slight fluctuation in the extracellular pool during neuronal firing, versus several orders of magnitude change in cytosolic Ca$^{2+}$. Indeed, in order to achieve success with 1.16B in cultured neurons, extracellular Ca$^{2+}$ had
Figure 1.16 – Alternative Ca²⁺-responsive Gd(III) CAs developed by Logothetis, Tóth, Angelovski, and coworkers. These agents are designed to have $K_d$ values ~ 1 mM for detection of extracellular Ca²⁺. In order to decrease $K_d$ from BAPTA, representatives of these agents A) bear amides instead of carboxylate binding groups, B) utilize EGTA rather than BAPTA, and C) use a modified Zn²⁺-binding domain with a less-rigid metal binding pocket.
to be discreetly buffered by introduction of EDTA to generate a detectable $\Delta[\text{Ca}^{2+}]$. It is generally held that for use \textit{in vivo} a CA must generate a $10\% \Delta r_1$ to be visible.\textsuperscript{17,107} And despite assistance from EDTA, 1.16B only decreased $T_1$ by $2\%$.

In attempts to mitigate these issues towards generating a detectable $\Delta r_1 \textit{in vivo}$, Logothetis and Tóth have utilized alternate Ca$^{2+}$-binding domains and investigated incorporation of their original agents into supramolecular scaffolds for combined $\tau_R$- and $q$-modulation. In the former approach, Angelovski and coworkers used an aminobis(methyleneephosphonate) moiety for selective Ca$^{2+}$ chelation.\textsuperscript{108} Although this agent displayed a $q$-dependent $38\% \Delta r_1$ upon Ca$^{2+}$ addition and a $K_d \cong 2.5 \text{ mM}$ suitable for extracellular applications, it did not generate measurable change in $T_1 \textit{in vivo}$ in response to electrical stimulation, instead showing constant contrast enhancement from fully-saturated extracellular probe. In the latter approach, Logothetis and coworkers have conjugated their agents onto liposomes, dendrimers, and silica nanoplatforms to achieve $\tau_R$-modulated $\Delta r_1$ of up to $420\%$.\textsuperscript{109-111} \textit{In vivo} work with these probes has shown improved retention over their small molecule counterparts, but no success in functional imaging of extracellular Ca$^{2+}$ flux.\textsuperscript{112}

1.6.3 MR methods of detecting Ca$^{2+}$ flux that do not Utilize Gd(III)

In addition to Ca$^{2+}$-responsive Gd(III) CAs, there are other MR methods of detecting physiological Ca$^{2+}$ flux worth mentioning here. The first is the MR chemical shift agent 5F-BAPTA (BAPTA which is fluorinated at the 5\textsuperscript{th} position on both aromatic rings).\textsuperscript{113} 5F-BAPTA can identify which metal binds the chelating domain by utilizing magnetic resonance spectroscopy (MRS) to monitor slight change in ppm of the fluorine peak associated with chelation of the different metals known to bind BAPTA. Because each metal has a unique associated ppm value,
5F-BAPTA can be used in systems containing multiple chelated metals, including an in vivo example.\textsuperscript{114} In an interesting multimodal approach, Angelovski and coworkers synthetically modified one of their Ca\textsuperscript{2+}-sensitive MR CAs with a trifluoromethyl group for MRS detection.\textsuperscript{115} This agent was able to detect metal chelation by both \textsuperscript{1}H and \textsuperscript{19}F MRI, but to date has not been tested in cellular or in vivo applications. Despite being an interesting research tool, 5F-BAPTA and its analogs still suffer from poor cellular uptake and, more importantly for in vivo applications, 3D MRS imaging techniques have much poorer spatial resolution than MRI (~1 cm\textsuperscript{3}), making its use largely impractical for most MI pursuits.

Another method for MR detection of Ca\textsuperscript{2+} flux is manganese-enhanced MRI (MEMRI). MEMRI monitors contrast enhancement from Mn\textsuperscript{2+}. With five unpaired electrons and a favorably long $T_{1e}$, Mn(II) probes relax water protons analogously to Gd(III) CAs, although with lower $r_1$ values owing largely to Gd(III)’s larger magnetic moment.\textsuperscript{116} MEMRI, monitors contrast enhancement from free Mn\textsuperscript{2+} and as a result is purely a preclinical MI modality, as free Mn\textsuperscript{2+} has a similar toxicity profile to Gd\textsuperscript{3+} (both of these ions are similar in size to Ca\textsuperscript{2+} and can occupy Ca\textsuperscript{2+} channels).\textsuperscript{117} MEMRI is able to monitor Ca\textsuperscript{2+} flux precisely because Mn\textsuperscript{2+} can be interact with Ca\textsuperscript{2+} channels. Specifically, Mn\textsuperscript{2+} is able to enter the cytosol of neurons via voltage-gated Ca\textsuperscript{2+} channels, but is not able to be effluxed through cellular Ca\textsuperscript{2+} exportation machinery.\textsuperscript{118} Thus actively firing neurons will accumulate intracellular Mn\textsuperscript{2+} and appear as contrast enhanced regions in MR images. MEMRI is a relatively easy imaging study, as the Mn\textsuperscript{2+} can be administered systemically and does not require invasive CA injection procedures. Aside from toxicity drawbacks (there is a very fine line between enough Mn\textsuperscript{2+} for contrast enhancement and immediate death to the animal), MEMRI has very poor spatiotemporal resolution – it takes time for the Mn\textsuperscript{2+}
to accumulate in neurons and the Mn$^{2+}$ cannot diffuse back out of no longer active neurons. This combination of attributes usually makes MEMRI only suitable for backup validation of neuronal activity in a general region that has already been investigated by other methods.

The last MR approach for Ca$^{2+}$ detection has been pursued by the Jasanoff Lab and to date is the only example of successful Ca$^{2+}$-responsive MR imaging in the literature. Jasanoff and coworkers employ iron oxide nanoparticles that change their $T_2$ relaxivity ($r_2$) in response to Ca$^{2+}$-induced aggregation ($r_2$ is also modulated by decreasing $\tau_R$). In their early work in this area, they drew direct inspiration from fluorescent protein Ca$^{2+}$ sensors, utilizing the same calmodulin/M13 binding pair for Ca$^{2+}$ detection. Thus the Ca$^{2+}$ sensor consisted of a mix of calmodulin-functionalized iron oxide nanoparticles and M13-functionalized nanoparticles. When the mixture encounters Ca$^{2+}$, calmodulin binds M13 and the nanoparticles form an aggregated network. These nanoparticles were designed with a Ca$^{2+}$-affinity in the intracellular range (as dictated by the $K_d$ of calmodulin) and needed further optimization for in vivo applications given that endosomal encapsulation is the endpoint for nanoparticles in biological systems.

With this in mind, Jasanoff and coworkers redesigned their sensor for extracellular Ca$^{2+}$ detection. While extracellular Ca$^{2+}$ flux is not as biologically relevant as the intracellular flux associated with discreet neuronal firing events, bulk changes in the extracellular pool can still be correlated to neuronal activity. Additionally, extracellular Ca$^{2+}$ flux tends to be slow and diffuse, with behaviorally relevant changes lasting for tens of seconds. Thus, if a CA could generate detectable $\Delta r_i$ ($i = 1,2$) in response to 100 µM to ~2 mM Ca$^{2+}$ flux, it would be well within the spatiotemporal resolution capabilities of MRI. This is exactly what Jasanoff and coworkers achieved. Their most recent nanoparticle Ca$^{2+}$ sensor utilizes the Ca$^{2+}$-dependent
synaptotagmin/phosphatidyl serine interaction. This machinery is used by neurons for release of neurotransmitter at the synapse and governs binding of neurotransmitter-containing vesicles to the inner leaflet of the plasma membrane. Importantly, the Ca$^{2+}$ affinity of synaptotagmin is appropriate for extracellular Ca$^{2+}$ flux (measured $K_d$ for their nanoplatform is approximately 500 µM). The nanoconstruct consists of lipid-coated iron oxide nanoparticles (the lipid mixture includes phosphatidylserine) which are aggregated reversibly in the presence of Ca$^{2+}$ by a synaptotagmin Ca$^{2+}$-binding domain dimer (Figure 1.17). Using this revised nanoconstruct, Jasanoff and coworkers were able to detect discreet consecutive $\Delta r_2$ in response to $\Delta [Ca^{2+}]$ in solution and, groundbreakingy, in the brains of living rats. When neurons in a region were stimulated by injections of K$^+$, the nanoconstruct depicted this as corresponding increases in $T_2$ contrast in the region. The construct enabled visualization of multiple stimuli over an hour long imaging time course. While the nanoconstruct CA reported on bulk changes in extracellular Ca$^{2+}$ that took several minutes to generate and dissipate, it remains the first and only successful Ca$^{2+}$-responsive $^1H$ MRI probe.
Figure 1.17 – Strategy used by Jasanoff and coworkers for successful detection of extracellular Ca\(^{2+}\) \textit{in vivo}. When synaptotagmin binds Ca\(^{2+}\), it can interact with phosphatidyl serine, leading to nanoparticle aggregation and a \(\tau_R\)-modulated boost in \(r_2\).
Chapter 2: Evaluation of Gd-DOPTA-Et in Rodent Seizure Models
2.1 Rationale for Validating Gd-DOPTA-Et \textit{in vivo}

This chapter focuses on \textit{in vivo} validation of Gd-DOPTA-Et and builds on the work of Dr. Zhidong Ma and Dr. Keith MacRenaris, who are responsible for chemical and \textit{in vitro} characterization of the agent.\textsuperscript{1} The goal of this work was to observe an increase in MR signal intensity from tissue exposed to Gd-DOPTA-Et in response to increased intracellular Ca\textsuperscript{2+}. This would have indicated that the agent was being activated by intracellular esterase cleavage and could then respond to increased neuronal activity, a first in Gd(III) CA agent literature. It must be restated here that an increase in contrast enhancement from Gd-DOPTA-Et was critical to proof of agent success, because both Gd-DOPTA in the Ca\textsuperscript{2+}-free state and Gd-DOPTA-Et still provide significant contrast enhancement (e.g. the agent does not have a dark off-state).

Thus tissue exposed to extracellular Gd-DOPTA-Et will still appear bright in MR images. The only way to generate increased contrast from Gd-DOPTA-Et during increased cytosolic Ca\textsuperscript{2+} flux is for the agent to be activated and responding to intracellular Ca\textsuperscript{2+}. Appropriate controls and histology would rule out neuronal cell lysis being responsible for increased contrast.

Although there are established means to increase intracellular [Ca\textsuperscript{2+}] in immortalized cell lines, these methods are not suitable for validation of an MR agent because: 1) they do not yield MR-detectable cytosolic Ca\textsuperscript{2+} and 2) their effect is not long lasting enough to perform MR imaging without resulting in cell death and possible detection of extracellular Ca\textsuperscript{2+} from lysed cells. Therefore Gd-DOPTA-Et was tested in animal models after it was observed to have improved cell uptake/retention over Gd-DOPTA. Unlike tissue culture, \textit{in vivo} models provide an opportunity to detect huge increases in intracellular [Ca\textsuperscript{2+}] that last for long periods of time: generation of prolonged seizure states. Seizures result from rapid, uncontrolled firing of neurons which necessitates influx of Ca\textsuperscript{2+} into the cytosol for instigation of action potentials.

There are many ways of generating seizures in the CNS, but for testing Gd-DOPTA-Et, three factors were critical for selecting the appropriate animal model. First, the seizures had to be long-lasting to detect in long scan times necessary for high resolution MR imaging. Second, the seizing had to occur
without concomitant physical seizing of the animal or all acquired images would be blurred from the motion and unanalyzable. Third, the seizure had to generate high enough intracellular Ca\textsuperscript{2+} levels to detect by MR and by activated Gd-DOPA (\(K_d \cong 1 \mu M\)). A fourth criterion was not necessary, but made experiments easier: systemic delivery of the seizure inducer or an intracranial delivery mechanism that was MR-compatible. Important to note here is that electrophysiological methods (which rely on implanting an intracranial electrode to the brain region of interest for direct electrical neuronal stimulation) can easily be made MR-compatible by using non-ferromagnetic components, but the readout instrumentation becomes very specialized and costly. In addition, implantation of electrodes in conjunction with stereotaxic agent delivery requires more CNS volume and usually is performed in rabbits and primates, both of which were beyond the scope of validating a new CA. For these reasons, chemical means of inducing seizures were selected over electrophysiological methods.

The class of seizure-inducing chemicals that meets the aforementioned criteria are excitotoxins. Glutamate is the major fast excitatory amino acid neurotransmitter in the CNS and is tied to Ca\textsuperscript{2+} signaling for instigation of action potentials at target neurons and for its release into the synaptic gap.\textsuperscript{2} Glutamatergic signaling is further linked to Ca\textsuperscript{2+} flux, because several types of glutamate receptors contain a ligand-gated Ca\textsuperscript{2+} channel domain.\textsuperscript{3} Activation of these “ionotropic” glutamate receptors triggers many immediate downstream events including membrane depolarization through the opening of voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+} channels, and release of additional excitatory neurotransmitter into the synaptic gaps.\textsuperscript{4-5} When glutamatergic signaling is perturbed by compounds that lock open ionotropic glutamate receptors, unregulated excitatory neuronal firing occurs.\textsuperscript{6-9} This hyperexcitation is perpetuated to downstream neurons via release of additional excitatory neurotransmitters and leads to toxic effects as neuronal energy stores
become depleted in the attempt to efflux ions back out of the cell. This phenomenon has been termed “excitotoxicity”. \(^4\) Researchers noticed early on that the cognitive and behavioral manifestations of excitotoxins are very similar to symptoms of seizure disorders. \(^10\) Since then, many other CNS conditions have been tied to abnormalities in glutamatergic signaling, including schizophrenia and bipolar disorder. \(^11\) Although the role of Ca\(^{2+}\) signaling in these non-acute neurodegenerative diseases is not well-understood, laboratory models of excitotoxicity remain the best option for studying acute CNS conditions such as epilepsy and ischemic stroke. \(^12\) Thus successful visualization of excitotoxicity by Gd-DOPTA-Et will shed more light on the temporal domain of excitotoxic neuronal damage.

Kainic acid (KA) is the most attractive excitotoxin for validating Gd-DOPTA-Et, because it displays high selectivity towards kainate and AMPA receptors in the hippocampus even when delivered systemically. \(^13\) The CA3 subfield of the hippocampus is particularly stimulated by KA since it is farthest downstream in the hippocampal circuit. \(^14\) - \(^17\) KA excitotoxicity leaves surviving neurons at CA3 in a perpetually hyperexcited state which proceeds without concomitant physical seizures. \(^13\) The former is ideal for long image acquisition times, while the latter improves image resolution by decreasing motion artifacts. KA is able to traverse the blood brain barrier after intravenous (IV) or intraperitoneal (IP) injection and generates predictable seizures that are well-characterized in the literature in rodents.

2.2 Cannulation Experiments in Rats

When in vivo experiments were discussed and planned, it was important to visualize temporal behavior between Gd-DOPTA-Et in order to visualize its activation by intracellular esterases. Gd-DOPTA-Et should decrease in \(r_1\) by 30% when it is cleaved to release Gd-DOPTA
in the Ca\textsuperscript{2+}-unbound state (Table 2.1). In addition to increase in signal after exposure to KA, an initial decrease in signal would be further evidence of proper agent function. For this reason, cannulated delivery of CA was selected as the best method to witness both agent activation and Ca\textsuperscript{2+} flux detection. Rats are larger than mice, making intracranial surgeries slightly easier, and were hence selected as the animal model.

<table>
<thead>
<tr>
<th></th>
<th>$r_{1, \text{Ca}^{2+}}$</th>
<th>$r_{1, \text{no Ca}^{2+}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd-DOPTA</td>
<td>12.5 ± 0.4</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>Gd-DOPTA-Et</td>
<td>10.2 ± 0.2</td>
<td>10.6 ± 0.3</td>
</tr>
</tbody>
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Table 2.1 – Relaxivity values for Gd-DOPTA and Gd-DOPTA-Et.

Delivery of material to the CNS through cannulae is well-precedented in the literature and is effectively implantation of a tube (“guide” cannula) to a specific brain region for later delivery of agent to the desired tissue. There are well-defined locations of brain regions in mammals with respect to intersections of the various cranial bones composing the skull. Thus, for a given brain region (say the CA3 subfield of the hippocampus), one need only consult a rodent brain atlas to determine where that region is with reference to skull features.\textsuperscript{18} As long as the animal being used is of standard age/size, the known coordinates of CA3 will hold true. The guide cannulae are implanted with the aid of a stereotaxic apparatus, which is a precision instrument that can measure and manipulate tools in 3D. After removing the tissue covering the top of the skull, the apparatus measures the appropriate $xy$ location of CA3 (the mediolateral and anterio/posterior coordinates, respectively, from a skull intersection point) and a burr whole is drilled through the skull at that location. Then the guide cannula is lowered slowly to the appropriate depth (the dorsoventral coordinate) and affixed to the skull with an adhesive (usually dental cement). A “dummy” cannula
is used to plug the guide cannula during healing to prevent tissue penetrating the guide. A hollow “injector” cannula replaces the guide once agent is ready to be injected.

Cannulation allowed for continuous monitoring of hippocampal signal intensity throughout agent injection/activation/response to excitotoxicity, because the animal could remain unmoving in the bore of the scanner. Since MR-compatible cannulae are readily commercially available and plastic tubing for agent delivery is non-ferromagnetic, the challenge was to devise a setup able to reliably deliver precise small volumes (up to 2µL) of CA through a long distance of tubing to reach the animal in the bore of the scanner.

2.2.1 Agent Delivery and in vivo MR Imaging Setup

To meet the needs of delivering µL volumes through tubing to an animal in the central bore of an MR scanner, a unique delivery setup was devised. The CA needed to be delivered over a period of several minutes to minimize neuronal tissue damage at the injection site, necessitating a precision metal syringe pump. Thus, the CA not only had to be delivered approximately 1 m into the bore of the scanner, but also through an additional 2 m of tubing to where the syringe pump could be safely placed outside the magnetic field of the scanner. However, filling 3 m of tubing, even narrow inner-diameter tubing, with CA solution gives rise to too much wasted CA to be sustainable for multiple imaging sessions. The first attempt to avoid CA loss was to load CA solution only into the last several inches of tubing (~ 100 µL). In mock CA delivery trials, this was not successful; displacement of µL volumes of air by the syringe pump did not generate enough pressure to deliver the CA.

The next setup investigated was to fill a portion of the tubing and the Hamilton syringes used by the syringe pump with oil (food grade vegetable oil), leaving a variable air gap in the
tubing between the oil and the CA solution. While this was an improvement over the first oil-free setup, it still did not accurately dispense µL volumes of the CA solution and it resulted in delivery of the specified volume all at once over a short period of time, rather than steadily over the desired injection timespan. Rather than delivering the CA solution at a constant rate (e.g. 100 nL/min), the syringe pump built up pressure by compression of the air bubble until enough force was generated to move the CA solution in one burst. This was visibly apparent. The air bubble size was varied, but its presence always yielded imprecise CA delivery in mockups.

The final agent delivery system removed the air bubble altogether, such that the entire length of the tubing and the Hamilton syringes were completely fluid-filled. The setup was prepared as follows (Figure 2.1). Food coloring was mixed into vegetable oil to generate a dark oil mixture. Polyethylene tubing (0.58 mm/23 gauge inner-diameter; Plastics1) was cut to ~ 3 m and loaded with 250 µL CA solution at one end. Avoiding any air bubble, oil was loaded continuously with the CA solution and the tubing filled with oil to push the CA solution to the other end of the tubing such that it was uncontaminated by the oil. A 50 µL Hamilton syringe fitted with a 22 gauge needle was loaded with oil and the tubing affixed over the end of the needle, again avoiding any air bubbles. Next the CA solution side of the tubing was affixed onto the injector cannula (28 gauge MR-compatible PEEK; Plastics1) and the syringe pump used to perfuse the inside of the injector with CA solution to avoid injecting an air bubble into the brain.

An open-design receive-only surface coil (20 cm inner-diameter; Bruker) was necessary to generate high resolution brain images while accommodating the vertical protrusion of the cannulae. The surface coil was affixed to the head of the animal securely with laboratory tape. Once the surface coil was in place, the perfused injector cannula was lowered into the guide and
Figure 2.1 – CA delivery system for the rat cannulation MR imaging experiments. A precision syringe pump was used to accurately deliver μL volumes of agent through many feet of tubing so that the animal could remain in the bore of the scanner throughout the injection/imaging time course.
secured with adhesive (Gorilla brand super glue) to prevent any movement of the injector and the animal was subsequently positioned in the bore of the scanner (Bruker 7T horizontal scanner equipped with a warm-water heated rat cradle and isoflurane anesthesia) and imaged. Throughout imaging, pulse rate was monitored to verify depth of anesthesia and maintained at approximately 50 beats per minute. For each injection, calipers were used to measure displacement of the oil/CA solution interface, which was determined to accurately depict injection volume owing to constant tubing inner-diameter.

Once animals were positioned in the central bore of the scanner, as visualized by 3-plane low resolution localizer images from both the scanner receiver coils and the surface coil, a high resolution 3D image of the entire head was obtained to visualize accurate cannula placement and to serve as a pre-CA baseline reference (GRE pulse sequence; 150 µm isotropic resolution). Next, a timecourse of four 1 mm thick coronal slice images were obtained surrounding the plane of the guide cannulae, such that both the left and right cannulae were in the same plane. These slices were obtained at 150 µm resolution and a new set of slices was generated every 2 s. The slices were obtained from 5 min prior to CA injection to 1 hr post injection. Animals received 2 µL injections of CA at differing concentrations over a 10 min injection period. Depending upon the quality of the injection data, several 100 µm 3D images were obtained after the slice timecourse for analysis of agent diffusion and retention. Data analysis was performed on Jim (Xnapse) and Image J (NIH) software with assistance from Prof. Daniel Procissi at the Center for Translational Imaging. Animals were sacrificed at the conclusion of MR imaging.
2.2.2 Cannulation Surgical Methods

All surgical procedures and animal handling were performed in accordance with an animal protocol approved by the Northwestern Animal Care and Use Committee. Male Sprague-Dawley rats (Charles River) aged 7 weeks were obtained and habituated to mild handling for ease of post-surgical procedures and to minimize animal stress. Prior to surgery, animal were weighed for accurate dosing of analgesic and to monitor post-surgical wasting. Anesthesia was induced by isoflurane and maintained at the surgical plane (verified by lack of recoil from a sharp toe pinch) throughout surgery. Once anesthetized, surgical site was prepped by shaving off fur and sequential scrubbing of the scalp with betadine and saline. Animals were placed into a bilateral stereotaxis apparatus (Kopf Instruments) equipped with rat tooth and ear bars and an isoflurane supply. Animals were kept warm during stereotaxis by a heating pad. After secure head placement in the apparatus was achieved, animals received a buprenorphine dose for post-surgical pain (0.03 mg/kg) and had ophthalmic ointment applied to prevent eye drying.

Standard sterile field techniques were used during stereotaxis. To remove the scalp and reveal the cranial sutures, the scalp was secured by a haemostat, lifted, and cut away in an approximately 1 cm² incision. The membranes atop the skull were removed and the surface of the skull cleaned and dried with hydrogen peroxide solution. Once the skull surface was dry, lambda and bregma were located and the animal positioned such that the two sites were in the same dorsoventral plane. Next, the mediolateral and anteroposterior coordinates of CA3 (± 3.6 mm/-3.8 mm from bregma, respectively) were measured and marked on the surface of the skull. Four burr holes (2 rostral to the marked CA3 coordinates and 2 caudal) were drilled partially through the skull and tapped for nylon screws (Plastics1) to provide an anchor to the skull. The screws
were coated with dental cement (Dentsply) and screwed into the threaded holes. Burr holes were then drilled at the CA3 coordinates bilaterally, taking care to avoid damage to the underlying brain tissue. A scalpel was used to make a slit through the meninges in the to prevent the guide cannula dragging the membranes down into the brain and causing damage to underlying tissue. MR-compatible guide cannulae (22 gauge PEEK threaded for dummy placement; Plastics1) were slowly lowered to the CA3 dorsoventral coordinate (-3.2 mm from bregma) over a period of 5 min to minimize neuronal tissue damage. Once the cannulae were lowered to the appropriate coordinates, dental cement was poured in to the exposed skull wound to cover the screws and guide cannulae bases. The dental cement was allowed to dry for several minutes, at which time the animal was removed from the stereotaxis apparatus and placed in a warmed cage to awaken from anesthesia. Dummy cannulae were screwed onto the guides and animals were observed for an hour and returned to conventional housing. Buprenorphine analgesic was administered every 24 hrs for the first 3 days after surgery to prevent undue animal suffering. Unless otherwise denoted, animals were allowed to recover 7 days prior to imaging and checked daily to insure proper placement of the dummy cannulae to maintain cannula patency for CA injection.

2.2.3 Cannulation Imaging Results and Discussion

In order to conserve Gd-DOPTA-Et, animals initially received Gd-DOPTA bilaterally. The goal was to observe replicatable CA diffusion bilaterally – essentially, one hemisphere could serve as the control for the other in terms of tissue inhomogeneity-based signal differences. This was to have minimized animal sacrifice and imaging time. It became apparent from the first animals imaged that diffusion of CA into the neuronal tissue surrounding the cannulae was going to be critical for image analysis and that this would be challenging to achieve. In dynamic contrast
enhanced (DCE) MR imaging experiments such as those planned with Gd-DOPA-Et, it is necessary to monitor changes in MR signal intensity (SI) over time, normalized to the background MR signal prior to injection of CA, thus

$$\frac{\Delta S_{\text{CA}}}{\Delta t} = \left( \frac{\Delta S_I}{S_{I\text{baseline}}} \right) / \Delta t \quad (2.1)$$

In order to accomplish this analysis, one needs to be able to define a region of interest (ROI) comprising voxels in which contrast enhancement due to the introduced CA is occurring. Selection of an appropriate ROI can be accomplished by several precedent ed methods, including manually with standard MR image processing software tools, automated via software packages, and via specialized programs that select any voxels which display ΔSI over the imaging time course. If the CA diffuses evenly into the surrounding neuronal tissue, it is relatively straightforward to select the ROI. However, if the agent is not diffusing into tissue well, ROI selection becomes problematic in light of the presence of the cannulae, the undelivered CA still in the injector cannulae, and possible uneven penetration of CA into intracerebral vesicles. All of these issues arose within the first several animals imaged, as well as a fourth factor that made ROI selection and ΔSI/t analysis almost impossible: $T_2$-shortening from the CA (Figure 2.2). Animals received 2 µL CA injections from 0.25-2 mM. Since the CA did not readily diffuse into the neuronal tissue surrounding the cannula tips, these CA doses resulted in enough Gd(III) present at the cannula tips to observe image darkening due to $T_2$-shortening effects. Although the image darkening was transient, it made ROI selection and ΔSI/t analysis almost impossible due to huge drop in MR SI near the dark regions. The initial SI increase, followed by sharp decrease due to $T_2$-shortening, and finally gradual SI increase due to diffusion and decreased [Gd(III)] prohibited
Figure 2.2 – Representative image of Gd-DOPTA showing poor diffusion into hippocampal tissue. The slices on the left (A) were acquired before CA injection, while the slices on the right (B) were acquired approximately 45 min post CA injection. The agent largely diffuses up the outer walls of the cannulae. $T_2$-shortening is observed as the dark region at the base of the cannulae, circled in red.
inclusion of the $T_2$-shortened regions into the ROI. BUT at the same time the CA did not diffuse far beyond the $T_2$-shortened voxels, with the result that there was not a good ROI to analyze. The goal of the cannulation MR imaging experiments hence transitioned to achieving proper CA diffusion into neuronal tissue in order to generate a reasonable ROI for $\Delta SI/t$ analysis.

Initial cannulation imaging experiments indicated pooling of CA both at the cannula tips and up along the sides of the guide cannulae. It was hypothesized that this was due to damaged neuronal tissue forming a “wound track” around the guide cannulae. Rather than diffusing into the tissue surrounding the cannula tips, CA was instead taking the path of least resistance and pooling in the cavity that appeared to have formed around the cannulae. A thorough literature search revealed only one publication (from the Jasanoff Lab) that utilized cannulae to deliver an MR probe into the rodent CNS.\(^{19}\) MR images in this article showed similar contrast enhancement along the sides of the guide cannulae, but to a much lesser extent. There were two experimental differences that could explain why Jasanoff and coworkers achieved better diffusion: 1) they injected into the striatum rather than the hippocampus and 2) the injector cannulae projected into the surrounding neuronal tissue beyond the edge of the guide cannulae. In comparison to the hippocampus, which is composed of several alternating layers of white and grey matter, both of which have different densities and permeabilities, the striatum is relatively homogeneous, which might promote more even spatial diffusion.\(^{20}\) However, KA for generation of intracellular $\text{Ca}^{2+}$ flux necessitated imaging in the hippocampus so modulating projection of the injector cannula was tried instead.

In order for the injector cannulae to project beyond the tip of the guide cannulae, the injectors must be sized for the specified projection and the guide cannulae must be implanted to a
shallower dorsoventral coordinate such that the projected injector reaches CA3 and not beyond it into undesired neuronal structures. Through this methodology, animals were implanted with guide cannulae to accommodate 0.5-1.5 mm injector projections and imaged via injection of Gd-DOPTA. While diffusion improved slightly for the longer injector projections, the issues of pooling and $T_2$-shortening were by no means alleviated enough to allow for thorough $\Delta SI/t$ analysis (Figure 2.3). The only animal to show adequate spatial diffusion for $\Delta SI/t$ analysis bore a 3 mm projection deep into the striatum, as the rat hippocampus is too close to the brain surface to accommodate a guide cannula for such a projection. As is was unclear whether diffusion was caused by the projection or the tissue homogeneity of the striatum, another cannula variable was next modulated to gain insight into its affect on CA diffusion: projection of the dummy cannula used to maintain cannula patency during surgical recovery.

Dummy projection was varied from 0.5-1.5 mm analogously to the injector projection experiments. The motivation for this approach was to possibly create a pocket at the base of the cannulae from which agent would then have time to diffuse spatially rather than run back up the sides of the guide cannulae. In these experiments the injector projection was reverted back to zero. Unsurprisingly, modulation of the projection of the dummy cannulae did little to alleviate poor diffusion, nor did it prevent flow back up the would track (Figure 2.4).

At this point, the only imaging experiment to show even moderately successful diffusion was the 3 mm projection. This scenario is analogous to implanting the guide cannulae and then immediately imaging without a recovery period for the animal to heal from surgery and for neuronal swelling to decrease at the injection site. However, if formation of scar tissue around the guide cannulae was responsible for creating a pocket around them which in turn led to decreased
Figure 2.3 – Representative image of Gd-DOPТА injected into the CA3 using an injector cannula projection. Image A) corresponds to pre-injection SI, while image B was obtained 65 min post injection of CA. The injector projection used in this animal was 1 mm. CA did not successfully diffuse into the neuronal tissue and $T_2$-shortening is still present at the cannula tip. In this animal the tubing slipped of the syringe, resulting in failure to inject in the right hemisphere.

Figure 2.4 – Representative image of Gd-DOPТА injected into the CA3 using a dummy cannula projection. The image in A) corresponds to 40 min post injection, B) was 55 min post injection, and C) was 70 min post injection. Diffusion largely occurred laterally and into the ventricles rather than into the hippocampus and there is some decrease in $T_2$-shortening. Interestingly, diffusion into the ventricular space led to decreased pooling around the guide cannulae.
diffusion into neurons at the injection site, eliminating the scar tissue might be a solution to the diffusion problems being faced. Increased swelling at the injection site would certainly be present due to elimination of the recovery period, but erroneous contributions to ΔSI/t arising from accompanying increases in hydration and cell lysis can be ruled out with proper controls and should not alter the large increase in intracellular [Ca^2+] upon exposure to KA. Therefore, animals were imaged immediately surgical implantation of the guide cannulae. Procedurally, these surgeries were much more straightforward, as they were non-survival and thus did not require maintenance of a proper sterile surgical field of administration of any analgesics – the animals remained at the surgical plane of anesthesia throughout surgery, transfer to the scanner, and MR imaging and were humanely sacrificed at the conclusion of imaging.

MR images of the non-recovery animals showed improved diffusion of Gd-DOPPA into tissue (Figure 2.5). However, $T_2$-shortening was still present at the cannula tips and the diffusion that did occur was not replicatable across hemispheres or different animals. Injector cannula projection was again varied but this did little to alleviate poor diffusion. In addition to $T_2$-shortening providing challenges to ΔSI/t analysis, CA diffusion often was more lateral than spherical, suggesting hippocampal tissue inhomogeneity played a significant role in CA diffusion. This was not as large an issue as $T_2$-shortening for ΔSI/t analysis, because 4 coronal slice images were acquired surrounding the plane of the cannulae and could be summed for analysis.

Although elimination of the recovery period improved diffusion, it did not yield analyzable MR images. In addition, assuming that KA yielded and increase in SI from Gd-DOPPA-Et, the recovery period would be necessary to monitor biologically relevant Ca^{2+} flux in future experiments, especially those with a temporal domain. It was clear that a more precise,
Figure 2.5 – Representative non-recovery images. The left and right columns correspond to different animals. The top and bottom rows are before and after injection of CA, respectively. C) and D) both show diffusion of CA into the ventricular space. While both animals also show some diffusion up the wound track and $T_2$-shortening, diffusion of Gd-DOPTA into the hippocampal tissue is improved significantly.
reproducible CA injection paradigm would need to be devised to evaluate response of Gd-DOPA-Et to KA-induced intracellular Ca\(^{2+}\) flux. Fortuitously, the cannulation work was presented to Prof. Daniel Dombeck (Northwestern Neurobiology) at a Neurobio Data Lunch talk. Collaboration with his lab provided the instrumentation and expertise to evaluate Gd-DOPA-Et in a more precise animal model: stereotaxic injection of CA through beveled glass micropipettes.

### 2.3 Microinjection Experiments in Mice

Stereotaxic injection through beveled glass micropipettes is much less disruptive to surrounding neuronal tissue than cannulation and was hypothesized to lead to replicable spatial diffusion of CA. This is a consequence of the size difference between cannulae and pulled glass pipettes. The smallest MR compatible injector cannulae available are 28 gauge (320 µm outer-diameter), while beveled glass pipettes taper to a tip that is a fraction of a micron in diameter! They are small enough to slowly push aside neurons rather than rupture them and are the standard minimally-disruptive intracranial injection method. Although injection removed the opportunity to visualize the entire CA injection/activation timeline, with appropriate experimental planning the gap between CA injection and MR imaging would be minimal (typically 40 - 60 minutes) and response to KA should not be impeded.

The Dombeck Lab (and countless other Neurobiology groups) relies on stereotaxic injection through glass micropipettes for transfection of fluorescent Ca\(^{2+}\)-sensing proteins to brain regions of interest for subsequent \textit{in vivo} optical neuronal functional imaging. Therefore, their stereotaxis setup is optimized to deliver small nL volumes of viral transfecting agent to a single brain region at a time rather than bilaterally. As a consequence, the maximum CA injection volume possible using the Dombeck Lab setup is 1 µL, although this is optimistic as the pipettes become
clogged very easily by any particulate present. In addition, Gd-DOPTA-Et requires several percent DMSO for solubility in aqueous media. DMSO adds to viscosity of the CA solution, further hampering injection of larger volumes. Thus the volume of CA injected was dictated by plugged pipettes and most injections were closer to 500 nL. The Dombeck setup is also only suitable for mouse surgeries. As a result of instrumental constraints, mice were used instead of rats, and animals received injection of CA in only one hemisphere. The uninjected hemisphere would serve as the effective baseline SI analogously to the pre-injection SI recorded in the cannulation experiments. Animal work in this section was accomplished with assistance from Mike Adoff in the Dombeck Lab under an approved animal protocol, and MR imaging/analysis was performed with the assistance of Prof. Chad Haney and Dr. Alex Waters of the Center for Advanced Molecular Imaging (CAMI).

2.3.1 Agent Injection an in vivo MR Imaging Setup

All stereotaxic microinjection experiments used male C57BL/6J mice (Charles River) aged 6-8 weeks. All surgeries were non-survival and animals were maintained at the surgical plane of anesthesia during surgery, transfer to the scanner, and MR imaging. Prior to surgeries, fresh glass pipettes were pulled, filled with CA solution, and beveled to a resistance of 2-4 MOhms (~ 0.5 µm diameter aperture) using standard techniques. An optical microscope was used to verify pipette tips were beveled and intact, as they are prone to cracks and fractures during the beveling process. Tips were also tested for clogs immediately prior to injections.

Before undergoing stereotaxis surgery, mice were weighed, anesthetized, and fitted with an IP catheter for later administration of KA (20 mg/kg). Placing the IP catheter prior to surgery minimized time between CA injection and MR imaging. The IP catheter was initially filled with
saline and periodically flushed to maintain patency. Mice were kept under anesthesia to prevent disruption of the catheter and immediately underwent stereotaxis surgery. After trimming away scalp fur, applying ophthalmic ointment, and washing the surgical site with saline, animals were secured in the stereotaxis apparatus (Kopf Instruments). A rectal thermometer and thermocouple heating pad were used to maintain the animal’s core temperature during surgery. Once the head of the mouse was securely locked into the apparatus, a ~1 cm incision made into the scalp to reveal the skull. Small haemostats were used to keep the edges of the scalp away from the injection coordinates and the surface of the skull was cleaned and dried with ethanol. The head of the mouse was positioned such that bregma and lambda were in the same plane. The coordinates of CA3 (1.8 mm lateral/2.4 mm caudal from bregma) were marked and a small burr hole drilled at that location. In order to minimize damage to the underlying neuronal issue, the burr hole was partially drilled with a surgical drill and the last microns on skull cracked and removed with sharp fine-tipped tweezers. The beveled glass micropipette was slowly lowered to the appropriate dorsoventral coordinate (~1.3 mm from bregma) over a period of 5 min. A pressure gauge was used to steadily inject up to 1 µL of CA solution (at a rate of ~ 25 nL/min) and reveal whether the pipette tip was becoming clogged. CA injection ceased at 1 µL or when pressure needed to inject further CA solution was >50 PSI. Once CA injection was stopped, the micropipette was slowly raised over a period of 3 min and dental cement (Dentsply) was used to seal up the burr hole and the exposed skull. Immediately after application of the dental cement, the animal was removed from the stereotaxis apparatus and transferred to a mobile anesthesia unit for transportation to CAMI and subsequent MR imaging.
Mice were imaged on a 9.4T horizontal scanner (Bruker) equipped with a warm-water heated mouse cradle and isoflurane anesthesia. Respiration and rectal temperature were monitored throughout imaging to insure proper depth of anesthesia. A mouse receive-only brain volume coil (Bruker) was employed for high resolution imaging of the brain. Once the animal was properly placed in the bore of the scanner, a fast $T_2$-RARE image was performed to locate the coronal slices surrounding the injection site. This volume was then imaged at 100 µm in-plane resolution, with new images obtained at 2 min intervals for ΔSI/t analysis. Interspersed with the kinetic data, 3D $T_1$-weighted GRE images were obtained to generate maps of agent diffusion into the hippocampal tissue. KA was injected through the IP catheter at varied time points and imaging continued until minimal contrast enhancement was observed in the injection region, corresponding to CA clearance. A slight change in respiration rate after KA injection was used to indicate neuronal seizing from hippocampal excitotoxicity. At the conclusion of MR imaging, mice were humanely sacrificed.

2.3.2 Results and Discussion

Use of beveled glass micropipettes dramatically improved diffusion of Gd-DOPTA-Et and Gd-DOPTA into hippocampal tissue (Figure 2.6). In addition, improved diffusion removed $T_2$-shortening. The only complicating factor in ΔSI/t analysis was from trace neuronal damage at the center of the injection site that arose at higher CA concentrations, but this was easily remedied by injecting less CA. Injection concentrations from 0.5 – 4 mM were explored; 2.5 mM was the maximal [CA] that did not lead to formation of a MR-visible lesion at the injection locus. Another approach to maximizing the amount of CA injected that was investigated in a couple animals was to make several different injections each at a slightly different location within the CA3 region.
Figure 2.6 – MR image of Gd-DOPA-Et injected through beveled glass micropipettes. The contrast-enhanced region contains the entire hippocampus and no $T_2$-shortening is observed at the injection site.
Although MR images of these animals showed a larger contrast-enhanced volume, each additional injection added a minimum 20 min between the first injection and MR imaging and this methodology was abandoned in favor of a single injection of 2.5 mM CA. To minimize chances of the glass micropipette clogging too early during the single injection approach, all CA solutions were filtered through a 20 µm filter prior to loading into the pipette.

ΔSI/t analysis revealed a difference in temporal contrast enhancement profile between Gd-DOPA-Et and Gd-DOPTA. For the same [CA] and injection volume, Gd-DOPA-Et produced less signal intensity and had a shallower slope in ΔSI/t compared to Gd-DOPTA (Figure 2.7). SI was normalized to brain tissue remote from the injection site. This is consistent with expected behavior. Gd-DOPTA should be saturated by mM extracellular Ca^{2+} and be in the fully bright on-state, while Gd-DOPA-Et is in the lower r₁ off-state until it is both cleaved by intracellular esterases and is subsequently exposed to MR-detectable intracellular Ca^{2+} generated by exposure to KA. Gd-DOPTA is also expected to diffuse more rapidly from the injection site if Gd-DOPA-Et is being taken up by neurons much more efficiently.

Once surgical and injection procedures were established in the initial non-KA experiments, the goal was to see if KA gave rise to an increase in SI from Gd-DOPA-Et. Five mice were used in these experiments. The first mouse received 1.75 nmol of Gd-DOPA-Et and was dosed with 20 mg/kg IP KA 360 min after the CA injection. It slowly died throughout the post-KA imaging time course, most likely from acute KA toxicity. Therefore, all subsequent mice received a decreased KA dose of 12 mg/kg. The second mouse received 1.49 nmol Gd-DOPA-Et and was dosed with KA 182 min after CA injection. The third mouse received 1.68 nmol of Gd-DOPA-Et and was dosed with KA 153 min after CA injection. The fourth mouse received 1.75 nmol Gd-
Figure 2.7 – ΔSI/t analysis for Gd-DOPTA and Gd-DOPTA-Et. The shallower slope of the Gd-DOPTA-Et linear regression corresponds to a slower washout rate from the hippocampus.
DOPTA-Et and was dosed with KA 171 min after CA injection. The fifth and final mouse received 1.75 nmol of Gd-DOPTA-Et and was dosed with KA 147 min after CA injection. The fourth and fifth mice received an additional 5 mg/kg KA booster approximately 20 min after the first KA dose in an attempt to make sure KA was generating hippocampal excitotoxicity.

ΔSI/t analysis revealed that KA Gd-DOPTA-Et failed to increase contrast enhancement in the hippocampus after systemic exposure to KA (Figure 2.8). Instead, SI continued to decrease in the hippocampus as Gd-DOPTA-Et diffused away from the injection site and contrast enhancement from Gd-DOPTA-Et had returned to baseline SI by 4 hrs post CA injection. Even though Gd-DOPTA-Et was more persistent in neuronal tissue than Gd-DOPTA, it did not get taken up in large enough quantities \textit{in vivo} for successful detection of intracellular Ca$^{2+}$ flux, as evinced by the 4 hr washout time. In order to successfully detect intracellular Ca$^{2+}$ flux, Gd-DOPTA-Et would need to be taken up by neurons in large enough amounts to still be visible after residual extracellular CA had diffused away from the injection site. This is a direct consequence of pre-activated Gd-DOPTA-Et still having a significant $r_1$ (due to outer-sphere effects discussed in Section 1.4.3). Accumulation of Gd-DOPTA-Et in neuronal tissue would have been indicated by a plateau in Gd-DOPTA-Et washout, rather than a constant decrease in SI over time until SI reached that of non-contrast-enhanced brain regions. Thus, regardless of its failure to detect KA-generated excitotoxic intracellular Ca$^{2+}$ flux, Gd-DOPTA-Et was simply not cell permeable enough for use in \textit{in vivo} applications.

\textbf{2.3.3 Outlook}

KA experiments with Gd-DOPTA-Et in mice clearly indicated that the agent was not taken up by cells efficiently enough for \textit{in vivo} use. If CA uptake could be improved, the same set of
Figure 2.8 – MR image of Gd-DOPTA-Et in the mouse hippocampus before and after exposure to kainic acid. Rather than responding to intracellular Ca$^{2+}$ flux, the CA rapidly diffused from the injection site. The red arrow indicates slight damage to the neuronal tissue at the injection locus, arising from concentrated CA solutions necessary for maximal SI and cellular uptake.
mouse experiments could be performed to determine whether CAs in the Gd-DOPTA family are capable of detecting intracellular Ca\(^{2+}\) flux. In order to accomplish this, a new agent was designed and synthesized (Gd-DOPTA-Fluor; see Chapter 3) which incorporated a cell transduction.

During the KA experiments in mice, it became clear that the KA model for generation of hippocampal excitotoxicity was not compatible with isoflurane anesthesia. KA exposure did not always generate changes in respiration rate (the only easily monitored indicator of physiological stress) and often the KA dosage that did lead to increased respiration rate was fatal. In addition, some mice died from a certain KA dose (20 mg/kg), while others showed no indication of neuronal seizures at the same dose. A thorough literature search revealed two root causes of the questions raised during the KA experiments. First, unlike in rats (the original animal model system), mice have a higher KA tolerance that varies with strain and is not consistent within a given strain.\(^{23}\) Coincidentally, the C57BL/6J strain used in this work is known for having such a high KA tolerance and variable response that it has been termed “KA-resistant,” with the only reliable method for generation of excitotoxicity being slow infusion of KA over long time periods to accurately pinpoint an individual mouse’s tolerance.\(^{24}\) However, the second source of failure to detect seizure signs is more significant and rules out use of KA in conjunction with isoflurane anesthesia – isoflurane and many other general anesthetics function by dampening neuronal firing. Although the exact mechanism of action is unknown, isoflurane has long been used clinically to stop epileptic seizures that are not managed by conventional therapeutics.\(^{25-27}\) It is ironic [and frustrating] that the clinical literature answers the isoflurane/KA question so succinctly when the pre-clinical literature supports seizure induction by KA under isoflurane anesthesia.\(^{28-29}\)
Due to inconsistencies between the clinical and pre-clinical literature, a collaborator (Prof. Hongxin Dong) provided an opportunity to definitively determine whether KA can induce seizures in mice under isoflurane anesthesia. A spare mouse with implanted intracranial electrodes was placed under isoflurane anesthesia and fitted with an IP catheter. KA was injected slowly over the course of 2 hrs. During this time, no changes in electrophysiological readings from the electrodes were observed. After administration of ~ 300 mg/kg KA (an order of magnitude higher than the literature-supported excitotoxic dosage for that strain), the mouse died from acute KA toxicity rather than excitotoxicity. Although this finding indicated that the KA experiments in mice did not in fact generate hippocampal Ca\textsuperscript{2+} flux (changes in respiration rate were instead likely due to stress from the mice being under anesthesia for long periods of time for IP catheter placement, surgery, and then MR imaging), they did not change the conclusions drawn from these experiments. Gd-DOPTA-Et still was not retained by cells well enough for \textit{in vivo} studies.

After consulting with Neurobiology experts, an alternate method of inducing seizures in immobilized mice was devised and validated in pilot animals. The solution was to use sedation during MR imaging rather than anesthesia. Specifically, medetomidine was chosen because it is also a highly-effective analgesic and would minimize chances that post-operative mice would experience pain during imaging.\textsuperscript{30} In this scenario, mice are under isoflurane anesthesia throughout stereotaxis surgery and CA injection, but are transitioned to heavy medetomidine sedation for MR imaging. The literature suggests that KA \textit{is} able to generate hippocampal excitotoxicity under sedation.\textsuperscript{31} Successful generation of seizures after transitioning from isoflurane anesthesia to medetomidine sedation was validated experimentally by electrophysiological recordings. For MR imaging purposes it was also determined that KA
seizures generated under medetomidine sedation do result in measurable changes in respiration rate and heartbeat. This animal model will be used for in vivo testing of future Gd-DOPA agents.
Chapter 3: Development of Gd-DOPTA-Fluor Contrast Agents
3.1 Rationale for Design of Gd-DOPTA-Fluor Agents

The goal of this Chapter was to develop a new Ca\(^{2+}\)-sensitive Gd-DOPTA agent bearing a cell-transduction domain to address poor retention of Gd-DOPTA-Et in vivo. There were three primary factors that contributed to the macroscopic design of the agent. First, it was important to pick a cell-transduction domain that would be compatible with high dosages required for MR CAs. This meant that the cell-transduction moiety selected needed to non-cytotoxic and have high solubility in aqueous media and consequently ruled out use of cationic amino acids (polyArg and polyLys) which are highly toxic in cellular systems and lipophilic cell-transduction domains such as trans-stilbene, respectively.\(^1\) Second, addition of a cell-transduction domain would ideally not add too many difficult and low-yielding steps to the overall synthesis of the agent. The goal was to design a modular Gd-DOPTA agent that could be conjugated late in synthesis to the cell-transduction moiety. The easiest route to achieve this was to conjugate the cell-transduction domain off of the Gd(III) macrocycles. Specifically, to modify one of the acetate arms such that it bore a compatible functional group for later conjugation to the cell-transduction domain. While modifying the acetate groups on the macrocycle lowers the stability of Gd(III) chelation, it still yields thermally stable ligands and is much easier to accomplish than modification on the macrocycle itself.\(^2\)\(^-\)\(^3\) The last factor that was considered in overall design of the new agent was esterification on the BAPTA domain. For ease of synthesis and in vitro characterization, the agent would not be esterified. In the Gd-DOPTA-Et experiments the failure of the agent was that it did not have high cell uptake, not that it was responsive to extracellular Ca\(^{2+}\). Successful detection of intracellular Ca\(^{2+}\) flux by Gd-DOPTA agents will necessitate prior clearance of extracellular CA, so masking the Ca\(^{2+}\)-binding domain was deemed unnecessary.
The cell-transduction domain that met the first criterion was the near-IR dye IR-783. The Meade Lab recently showed that conjugation to IR-783 can improve uptake of high molecular weight Gd(III) compounds by orders of magnitude, achieving uptake in cultured cells as high as 400 fmol/cell.\(^4\) For calibration, Gd-DOPTA-Et was only taken up by cultured cells in amounts ≤ 8 fmol/cell. It is hypothesized that conjugation to IR-783 facilitates cell uptake due to targeted uptake of the dye by organic anion transporter proteins (OATPs).\(^5-6\) Several isoforms of the OATP family have high expression levels in the brain, suggesting that this is a viable strategy for increasing agent uptake in cells of the CNS.\(^7\) In addition, conjugation of Gd-DOPTA to a near-IR dye (Gd-DOPTA-Fluor) presents the opportunity for multimodal imaging: the dye serves as a tracer for optical validation of agent localization. The target Gd-DOPTA-Fluor structure (Figure 3.1) utilized literature-precedented IR-783 moiety bearing an isothiocyanate (“IR-783-SCN”) for conjugation to a literature-precedented arm-modified macrocycle bearing an amine.\(^8-9\) Upon completion of synthesis, minimal testing in tissue culture would reveal whether conjugation to IR-783 improved cellular uptake. If so, Gd-DOPTA-Fluor would be subjected to streamlined evaluation in the mouse model discussed above, building on the aspects of experimental design learned through trial and error with Gd-DOPTA-Et.

### 3.2 Symmetric Gd-DOPTA-Fluor Agents

#### 3.2.1 Synthetic Progress Towards Symmetric Gd-DOPTA-Fluor Agents (e.g. Failed Routes)

The initial proposed Gd-DOPTA-Fluor agent shown in Figure 3.1 was symmetric about the BAPTA core in order minimize number of synthetic steps and to avoid a costly yield hit from another asymmetric synthetic step. The synthetic route shown in Scheme 3.1 was devised and attempted. This route had a total of 18 steps, 10 of which were linear, and would yield two Gd-
Figure 3.1 – Proposed Gd-DOPTA-Fluor structure. The IR-783 cell transduction domain moiety is shown in green.

Scheme 3.1 – Proposed synthesis of symmetric Gd-DOPTA-Fluor structures. This route uses three basic building blocks: brominated BAPTA core (1), Cbz-amino modified DO3A macrocycle (2), and IR-783-SCN (3).
DOPTA-Fluor agents: a 1-dye analog and a 2-dye analog, both of which would be evaluated for cell permeability. However, only the later reactions denoted by asterisks were not previously reported in the literature. Acid-labile carboxyl protecting groups (e.g. tert-butyl esters) were selected because their cleavage in formic acid is less likely to yield degradation products than corresponding cleavage of base-labile ethyl esters by concentrated hydroxide (anecdotal). The synthesis depicted in Scheme 3.1 utilizes three basic building blocks: brominated BAPTA core (1), Cbz-amino modified DO3A macrocycle (2), and IR-783-SCN (3). Synthesis of the BAPTA core proceeds according to the original reported Gd-DOPTA route, in an overall yield of 7% over 5 steps (see Scheme 1.1 for synthesis of the ethyl-esterified analog).¹⁰

The modified macrocycle is synthesized from cyclen and Nε-Cbz-L-lysine in a total of 6 steps (Scheme 3.2). Diazo chemistry is used to convert the lysine α-animo acid into an α-bromo acid, which is then esterified by t-Bu-2,2,2-trichloroacetimidate in 54% yield over 2 steps to yield Nε-Cbz-D-lysine α-bromo ester (5).⁹ t-Butyl protected DO2A (4) is obtained from cyclen in 3 steps at 71% yield.¹¹ In the first step, cyclen is selectively Cbz-protected at the 1 and 7 position nitrogens due to solvent effects (the 1,7-Cbz product is insoluble in chloroform so further reaction to the tri/tetra-Cbz products does not occur). Next, the 4 and 10 position nitrogens are alkylated with t-butylbromoacetate, followed by catalytic hydrogenolysis to remove the Cbz groups. The t-Bu-DO2A is then monoalkylated by the α-bromo ester to yield the modified macrocycle in 41% yield.⁹

IR-783-SCN (3) is obtained from conjugation of an isothiocyanate moiety to commercially available IR-783 chloride in 34% yield over 2 steps (Scheme 3.2). First, 4-amino-phenol is readily converted to 4-isothiocyanate-phenol under mild conditions via reaction with CS₂ followed by
Scheme 3.2 – Synthesis of the building blocks used in Scheme 1. Cbz-protected modified macrocycle (building block 2) is obtained from monoalkylation of t-Bu-DO2A with the α-bromo ester arm. IR-783-SCN is formed from substitution off of the commercially available dye chloride.
cleavage of the formed dithiocarbamate intermediate by p-tosyl chloride. Reaction of the 4-isothiocyanophenol with the dye-chloride using sodium hydride as a non-nucleophilic base yields 3.

The three building blocks (1, 2, and 3) were readily obtained, owing to their precedent in the literature. Once they were in hand, the BAPTA core was to be di-alkylated with the modified macrocycle. Sequential deprotection of the Cbz groups and t-bu esters, followed by metallation and conjugation to the dye would yield the 1-dye and 2-dye symmetric Gd-DOPTA-Fluor agents. In order to purify the fully-protected alkylation product 6, the reaction on the BAPTA core ideally needed to proceed to completion with use of excess macrocycle, such that the crude product contained primarily 6 and residual macrocycle. It is widely known by research groups working with macrocyclic Gd(III) CAs, that protected macrocyclic derivatives are very hard to separate using normal-phase chromatography (on both SiO\textsubscript{2} and Al\textsubscript{2}O\textsubscript{3} solid phases). Once a protected intermediate bears a macrocycle, the macrocycle seems to dominate behavior on column chromatography. Thus, most CA syntheses are designed either to go to completion from the standpoint of the macrocycle (which is readily purifiable from the alkylator), or to alkylate and carry the crude product directly into deprotection, metallation, and reverse-phase HPLC purification of the final chelate.

Neither one of these approaches was applicable to synthesis of Gd-DOPTA-Fluor in Scheme 3.1, as the BAPTA core was the costliest building block and there were too many steps before HPLC purification to realistically carry through crude product. However, 6 and 2 were hypothesized to be separable using appropriate chromatography conditions, owing to the huge differences in their molecular weights and number of polar functional groups. Indeed, these two
components of the crude mixture were separable on silica gel columns using very polar mobile phases (MeOH/DCM and MeCN/H$_2$O solvent systems). The problem was that they were not separable from monoalkylated byproduct and degradation products that arose during alkylation reactions of the BAPTA core. In all reaction conditions investigated, degradation (primarily degradation of the Cbz group, but also some loss of iminoacetate arms, among other side reactions) occurred at a faster rate than completion of alkylation. This was observed on electrospray ionization mass spectrometry (ESI MS) and thin layer chromatography (TLC). Even with huge excess of the macrocycle, the dialkylated product was inseparable from the other crude byproducts. The crude mixture was also not resolvable using reverse-phase high-performance liquid chromatography (HPLC) methods because it was too non-polar to properly elute from all of the HPLC stationary phases investigated, even in the presence of acidic mobile phase additives.

At this point, a modified synthetic route was devised to overcome issues with degradation during the reaction conditions necessary to completely alkylate the BAPTA core (Scheme 3.3). In this approach, the brominated BAPTA core was instead first dialkylated with $t$-Bu-DO2A (which was hypothesized to proceed with minimal degradation and eliminated the primary source of degradation: the Cbz group), then dialkylated with the Cbz-protected $\alpha$-bromo ester. The subsequent steps followed those outlined in Scheme 3.1. Although this route had an extra linear step (11 total), almost all of the reactions were the same as the previous route owing to the modularity of the building blocks and the overall number of steps (18) remained unchanged. As was hypothesized, the alkylation of the BAPTA core by $t$-Bu-DO2A proceeded with minimal formation of degradation products.
Scheme 3.3 – Modified synthesis of symmetric Gd-DOPTA-Fluor agents aimed at avoiding degradation observed during alkylation of the BAPTA core by the modified macrocycle. Compounds 4 and 5 refer to t-Bu-DO2A and the Cbz α-bromo ester arm, respectively.
Unsurprisingly, however, challenges arose during purification of the dialkylated intermediate 7 (Scheme 3.3). This intermediate and residual \textit{t}-Bu-DO2A both have two unmodified \textit{2\textdegree} nitrogens, which interact more strongly with the stationary phase in normal-phase chromatography than their modified \textit{3\textdegree} counterparts. Anecdotally, \textit{t}-Bu-DO2A does not readily elute from silica or alumina columns unless one resorts to flushing the column with large percentages MeOH or THF. The hope was that intermediate 7 would be rendered much less polar than \textit{t}-Bu-DO2A by means of the lipophilic BAPTA core. However, this was not the case. Under all chromatography conditions investigated (\textit{SiO\textsubscript{2}}/\textit{Al\textsubscript{2}}O\textsubscript{3}; MeOH/DCM and MeCN/H\textsubscript{2}O solvent systems with additives), the alkylated intermediate failed to elute from the column until the polarity of the mobile phase was high enough that it then streaked off inseparably from the mono-alkylated intermediate and \textit{t}-Bu-DO2A. Usually by this time the crude material had been on the column for long enough that the post-chromatography crude was contaminated by additional degradation products observable by ESI MS. Intermediate 7 was now \textit{too polar} for normal phase chromatography. However, it was still nowhere near water-soluble even with acidic additives and showed poor elution on reverse-phase HPLC columns.

In desperation, a batch of crude was submitted to Arsen Gaisin, then the compound purification specialist in the Center for Molecular Innovation and Drug Discovery at Northwestern. He was able to purify a small amount (46\% yield with respect to the BAPTA core) of the dialkylated product, which was subjected to dialkylation by a huge excess of the Cbz-protected \textit{\alpha}-bromo ester arm. The goal was to force the reaction to completion as fast as possible in mild conditions and thereby out-pace degradation. This strategy was successful. Intermediate 6 was readily purified by silica gel chromatography, as it was the only polar component of the mixture.
and minimal degradation was observed (ESI MS). The only remaining steps were deprotection of
the Cbz groups and 1-butyl esters, metallation with Gd\(^{3+}\), and dye conjugation. Catalytic
hydrogenolysis of the Cbz groups was chosen to be performed first so that deprotection could be
readily monitored by ESI MS (esterified macrocycles ionize several orders of magnitude better
than their deprotected counterparts). In addition, formic acid de protections of 1-butyl-protected
macrocycles are well-understood in the Meade Lab and cleavage of the esters was expected to be
facile.

With this in mind, intermediate 6 was subjected to standard Cbz deprotection conditions
(H\(_2\), Pd/C in EtOH).\(^{13}\) The reaction was monitored by TLC and ESI MS, and by 18 hrs all 6 was
consumed, but expected m/z values corresponding to partially deprotected intermediate 8 were not
observed, and the m/z values that were present were not able to be identified as salt adducts or
other fragments of 8. It was hypothesized that the deprotected primary amine was generating
unpredicted charged species in ESI, since the observed peaks in the mass spectrum were within
200 m/z of those expected from 8. In order to avoid complications from ionization and get a better
handle on whether deprotection was successful, matrix assisted laser desorption/ionization
(MALDI) MS was also performed on the crude. Unlike ESI, MALDI is a “soft” ionization
technique and usually generates spectra containing minimal fragments for ready visualization of
the molecular ion and its simple sodiated/potassiated charge adducts. However, MALDI generated
similar peaks to those observed in ESI MS and no product m/z were detected. It was assumed that
the discrepancies in both ESI and MALDI MS arose as a consequence of ionization and the formic
acid deprotection was carried out. Once the 1-butyl esters were cleaved, intermediate 9 was no
longer detectable by ESI MS. When reaction progress was monitored by MALDI MS, something
very strange was observed: the m/z present corresponded to losses of various t-butyli groups (including full ester hydrolysis) plus 2 Cbz groups! Clearly, the Cbz deprotection had failed.

In order to achieve successful Cbz deprotection, the first alternative tried was to reverse the order of protecting group cleavage, since the de-esterified Cbz product was readily visible by MALDI MS. Cbz cleavage by catalytic hydrogenolysis should be very straightforward and human error simply needed to be ruled out. Thus, intermediate 6 was subjected first to formic acid ester hydrolysis and then catalytic hydrogenolysis (H2, Pd/C in EtOH). After hydrogenolysis, MALDI again failed to show predicted m/z for the starting material or the fully deprotected intermediate 9. When the crude deprotection mixture was metallated with Gd3+, MALDI indicated that the Cbz groups were still present. Clearly, standard Cbz deprotection conditions were not working. Next, harsher Cbz cleavage conditions were attempted on small scale reactions on freshly prepared intermediate 6. Catalytic hydrogenolysis with Raney nickel and acid hydrolysis (BBr3, TFA, and HBr/HOAc) all led to decomposition of the molecule. An alternative to Cbz protection of the modified macrocycle arm was, unfortunately, necessary.

Cbz-protected Nε lysine was selected as starting material for the modified macrocycle arm because its use was precedented in the literature. However, having to separate deprotection steps was not necessarily the most streamlined synthetic route; one universal acid or base deprotection step (depending on incorporation of acid- or base-labile esters on the BAPTA core and the macrocycle) would be preferable. An acid-labile protecting group would need to withstand the oxidizing/acidic diazo conditions necessary to instill the α-bromo acid, and very few base-labile nitrogen protecting groups are compatible with the basic conditions required for many of the S_N2 alkylation steps. In addition, commercially available sources of Nε-protected lysine have limited
selection of protecting groups. Thus, rather than starting from alternately protected Ne lysine, attempts were first made to exchange the Cbz group for an acid-labile group. Boc protection was selected, because Boc groups are easy to install and are readily cleaved by formic acid. To save time, the protecting group exchange was first pursued on stockpiled modified macrocycle (Scheme 3.4). This approach still had 18 total steps, but fewer linear steps (9). Interestingly, Cbz deprotection on the macrocycle proceeded very smoothly, unlike at later stages of synthesis as previously observed. Upon completion of Cbz cleavage, Boc protection was performed under kinetic conditions to avoid protection of the 2° cyclen nitrogen. Although di-Boc-protected macrocycle was observed on ESI MS and TLC, mono-Boc-protected macrocycle 10 was successfully isolated and reacted with brominated BAPTA core. Exchange of the Cbz groups for Boc groups led to much cleaner alkylation of the BAPTA core and the alkylated intermediate 11 was purifiable by silica gel chromatography in low yields. The alkylated intermediate was then subjected to universal formic acid deprotection and metallated with Gd$^{3+}$.

However, an analytical HPLC trace of the crude metallated penultimate intermediate 12 revealed an unwelcome [but in hindsight very predicable] issue: there were 3 unresolvable peaks on the chromatogram that all had the target m/z. This is because nitrogens are not easily subjected to kinetically/sterically controlled reaction conditions. Instead, they react according to nucleophilicity and 2° nitrogens are more nucleophilic than 1° nitrogens. Thus, rather than intermediate 10 only displaying the desired Boc-protection on the modified acetate arm, the 2° cyclen nitrogen was also protected, such that 10 was actually a mixture of arm-protected macrocycle and cyclen-protected macrocycle (corresponding to a mixture of different connectivities in 12). Several reasons explain why the mixture was not detected during NMR and
Scheme 3.4 – Proposed exchange of protecting groups on the modified macrocycle using kinetic conditions for selective 1° nitrogen protection. 1 refers to the brominated BAPTA core.
MS characterization of 10. The difference in protection location of the two isoforms would not be detectable by MS, as they have the same molecular weight. It is also not surprising that chromatography only indicated presence of one species: as discussed previously, presence of the macrocycle dominates retention of small molecule compounds in normal-phase chromatography. For example, the Cbz deprotection step was monitored in part by normal-phase TLC, and both the starting material and the deprotected product (which bears a free 1° nitrogen) had similar RF values. Lastly, 1H and 13C NMR spectra of asymmetric macrocycles contain a large number of signals with very similar chemical shifts and are challenging to analyze. Given that intermediate 10 also had a long alkyl chain with many signals, the NMR spectra were not readily conducive towards indicating a mixture of products. This issue is even more challenging with the addition of more signals in intermediate 11. In contrast, the ESI mass spectra for 10 and 11 were very clean. Thus, although the NMR spectra do have slight anomalies, they were only noticed with the assistance of hindsight and presence of different product isoforms was only detected during HPLC of 12.

In order to avoid protection of the 2° cyclen nitrogen, a slightly different route towards conversion of the arm protecting group from Cbz to Boc was attempted (Scheme 3.5). This method utilized Fmoc protection of the cyclen nitrogen to selectively protect the arm nitrogen. Although the Fmoc protected intermediate was able to be isolated, the Fmoc group was not stable towards hydrogenolysis of the Cbz group. Most likely the deprotected 1° nitrogen on the modified arm was basic enough to cleave it.

The final attempt at utilizing Nε-Cbz-lysine derived α-bromo ester arm was to exchange protecting groups before alkylation of the α-bromo ester onto t-bu-DO2A. Several
Scheme 3.5 – Proposed exchange of protecting groups on the modified macrocycle using utilizing Fmoc protection of the cyclen 2° nitrogen to avoid a mixture of protected isoforms. Once deprotected, the 1° amine cleaved the Fmoc group, rendering this method unsuitable.
conditions to exchange protecting groups were tested, both at the α-bromo acid stage and the α-bromo ester stage. None of them were successful (α-bromo carbonyl compounds are not stable towards reductive conditions or harsh acidic/basic/nucleophilic conditions necessary for cleavage of Cbz groups) and will not be discussed further.

All of the synthetic work performed to this point was based on use of Cbz-protected α-bromo ester starting material for the modified macrocycle arm. Although Nε-Boc-L-lysine is commercially available, its use was not initially pursued for sound reasons, including lack of literature precedent and necessity of harshly acidic diazo conditions to instill the α-bromo acid in the first step of making the α-bromo ester arm. It was doubtful that the Boc group would be stable to 1 M HBr/NaNO₂. A quick test was in order: could Nε-Cbz-L-lysine be converted to the α-bromo acid in milder acidic conditions? The answer was yes, although in lesser yields. With this in mind, Nε-Boc-L-lysine was obtained and subjected to diazo conditions with varying [HBr]. 1 M HBr did in fact largely deprotect the Nε-Boc group, leading to dibrominated product observed in ESI MS. Of the [HBr] tested, 0.1 M HBr led to highest yield of α-bromo ester (12% over 2 steps). Even though higher [HBr] yielded greater conversion to the α-bromo acid, by products from the diazo reaction in these conditions were inseparable from the α-bromo acid and led to decreased yield in the esterification step, even using a huge excess of the t-Bu-2,2,2-trichloroacetimidate esterification reagent. Alternate esterification conditions were attempted, but all were unsuccessful (isobutylene/H⁺cat, t-BuOAc/TEA, t-BuOAc/HO⁻) or cleaved the Boc group (SOCl₂ followed by t-BuOH/TEA, isobutylene/HClO₄, isobutylene/H₂SO₄).

Although very low yields of Nε-Boc-L-lysine α-bromo ester were obtained, many large-scale reactions eventually yielded enough α-bromo ester arm to incorporate it into synthetic
Scheme 3.6 – Incorporation of Boc-protected α-bromo ester arm into synthesis of the symmetric Gd-DOPTA-Fluor. 1 refers to the brominated BAPTA core.
progress towards Gd-DOPTA-Fluor (Scheme 3.6). Use of Nε-Boc-D-lysine starting material removed the Cbz deprotection step, for a total of 17 steps, 9 of which were linear. Monoalkylation of the α-bromo ester arm onto t-Bu-DO2A was accomplished in 58% yield. Dialkylation of the brominated BAPTA core with the Boc modified macrocycle proceeded to completion with minimal degradation (analogously to Scheme 3.4). However, purification of the dialkylated intermediate 11 on silica gel was very challenging and very low yielding (≤ 14%), a very undesirable combination towards the end of a synthesis. For these reasons, a slight change in the synthetic plan was made, following that outlined in Scheme 3.3 – alkylation of the BAPTA core with t-Bu-DO2A utilizing reverse-phase HPLC purification, and then alkylation with the α-bromo ester arm. In addition, a new route incorporating base-labile ethyl esters on the BAPTA core and the modified macrocycle, as well as a base-labile trifluoroacetyl protecting group on the modified macrocycle arm, was pursued owing to low yields of intermediates 11 and 13 in Scheme 3.6. Both of these routes ultimately yielded the symmetric Gd-DOPTA-Fluor agents and will be discussed in detail in the next section. Fortuitously, at this point a new procedure for converting α-amino acids into α-bromo acids was found in the literature and, with a slight modification to decrease acidity during diazo reaction, yielded the desired Nε-Boc-L-lysine α-bromo ester in much greater yield (43% over 2 steps; see Scheme 3.7).\(^{14}\)

### 3.2.2 Final Synthesis of Symmetric Gd-DOPTA-Fluor Agents

The final, successful routes to the symmetric Gd-DOPTA-Fluor agents are shown in Scheme 3.7 and Scheme 3.8. Both of these routes have a total of 17 steps, (10 of which are linear) and utilize acid-labile and base-labile protecting groups, respectively. For ease of interpretation these schemes will begin a new numbering system distinct from Section 3.2.1 and the structures
Scheme 3.7 – Final synthesis of the symmetric Gd-DOPTA-Fluor agents utilizing acid-labile protecting groups. 1 refers to the brominated BAPTA core.
Scheme 3.8 – Final synthesis of the symmetric Gd-DOPTA-Fluor agents utilizing base-labile protecting groups.
of the final compounds are found in Figure 3.2. The total yield in both approaches is < 1%, largely owing to low-yielding HPLC purification steps which were not well-optimized. In Scheme 3.7, t-Bu-DO2A, IR-783-SCN, and intermediate 1 all followed procedures/purification obtained directly from literature precedent, while in Scheme 3.8, Et-DO2A, IR-783-SCN, and intermediate 1’ directly followed the literature. Final products 9 (1-dye symmetric Gd-DOPPA-Fluor analog) and 10 (2-dye symmetric Gd-DOPPA-Fluor analog) were the common endpoints to both approaches.

The most challenging aspect of the final synthetic routes was purification of the later intermediates (2-4, 2'-4', and 5), as well as the final products. Until the deprotection step, the intermediates were very high molecular weight and contained a huge number of polar functional groups, making them largely incompatible with normal-phase chromatography methods. However, as discussed above, they were still too nonpolar to readily be purified using reverse-phase HPLC methods. Once deprotected, the intermediates had multiple charge states, requiring additives for HPLC purification. Initially, many attempts were made to HPLC purify the metallated penultimate intermediate (5) rather than the free ligand (4/4’), following standard protocols for purification of Gd(III) compounds. However, the free amines on intermediate 5 led to multiple product peaks on the chromatogram during HPLC purification without additives. Basic additives led to poorly resolved peaks and, while acidic additives led to well-resolved peaks, the [acid] necessary to resolve the product peak led to partial demetallation of the Gd(III) chelates. In addition, the small amounts of 5 successfully isolated under neutral HPLC conditions were prone to degradation during concentration.
Figure 3.2 – Complete structures of the symmetric Gd-DOPA-Fluor agents.
HPLC purification of the free ligand 4/4' after deprotection, although not the standard approach, proved to be straightforward with acid additives. Although 4/4' were not water soluble (even with acid), they were soluble in acidic H$_2$O/MeCN mixtures at low enough percentage MeCN (15%) to separate well on reverse-phase C18 HPLC columns. Then, metallation proceeded relatively cleanly and the crude metallated intermediate 5 was able to be used without further purification in the last step (dye conjugation). Although the dye conjugation reaction was not itself optimized (it used the same solvent conditions as previously reported in the Meade Lab), carrying through crude penultimate intermediate might have also played a role in the extremely low yield observed for the final step. HPLC purification of the 2-dye Gd-D OPTA-Fluor agent (10) was relatively straightforward using basic additives. The product eluted in a somewhat sharp peak that displayed close to baseline resolution from similarly-retained impurities.

HPLC purification of 9, however, was very challenging, most likely due to the remaining free amine. This was problematic, as compound 9 was the desired Gd-D OPTA-Fluor agent, while compound 10 was expected to be purified as a by-product and was less-favorable because it had a higher molecular weight and an extra dye. Initial HPLC purifications of the dye-conjugation reaction (both analytical and semi-preparative) did not display a peak with appropriate m/z for the 1-dye final product 9, despite 9 being observed in MALDI spectra of the crude. Analytical HPLC-MS using acid additives displayed a significant peak corresponding to demetallation of 9, indicating that 9 was present but not well-resolved in standard basic conditions (0.1% NH$_4$OH). As a last effort to isolate enough 9 to perform relaxometric characterization, a higher concentration (1%) of basic additive was used and this method ultimately yielded ~ 3 mg of pure 9, despite wildly inconsistent HPLC chromatograms and small amounts of degradation during purification.
Challenges in purification of the 1-dye Gd-DOPTA-Fluor agent 9 were the motivation for the Gd-DOPTA-Fluor analog discussed in Section 3.4.

Unless otherwise indicated, all reactions were performed under a nitrogen atmosphere in oven-dried glassware. Anhydrous solvents were used in all reactions and obtained from a J.C. Meyer solvent system (Laguna Beach, CA). Thin-layer chromatography (TLC) was performed on EMD 60 F254 silica gel plates. Standard grade 60 Å 230–400 mesh silica gel was used for normal-phase column chromatography. Unless otherwise stated, all silica gel columns were flashed with air. $^1$H and $^{13}$C NMR spectra were obtained on a Bruker 500 MHz Avance III NMR spectrometer. MALDI MS was performed on a Bruker AutFlex III, using 2,5-dihydroxybenzoic acid as the matrix. ESI MS was performed on a Bruker AmasonSL spectrometer. Cyclen was obtained from Strem Chemical, while all other reagents were purchased from Sigma Aldrich or Fisher Scientific and used without purification. Analytical HPLC-MS was performed on an Agilent 1260 Infinity II HPLC system with an in-line Agilent 6120 Quad mass spectrometer. Semi-preparative HPLC was performed on an Agilent PrepStar 218 equipped with an Agilent 1260 Infinity diode array detector. HPLC purifications utilized deionized water (18.2 MΩ·cm) obtained from a Millipore Q-Guard System and HPLC grade MeCN, formic acid, and ammonium hydroxide (all obtained from Fisher Scientific). Analytical HPLC used either a Waters Atlantis C18 column (4.6 × 250 mm, 5 μm) or an XBridge C18 column (4.6 × 150 mm, 5 μm). Semipreparative HPLC used either a Waters XBridge C18 column (19 × 250 mm, 10 μm) or a Phenomenex Synergi Polar RP column (21.2 × 150 mm, 4 μm).

Compounds were purified using one of the following methods. Method A: MeCN held at 15% for 5 min followed by a 5 min ramp to 35% and a 15 min ramp to 45%. Method B: MeCN
held at 0% for 5 min followed by a 2 min ramp to 15% and a 23 min ramp to 35%. Method C: MeCN held at 0% for 5 min followed by a 2 min ramp to 15% and a 33 min ramp to 35%. Method D: MeCN held at 0% for 5 min followed by a 15 min ramp to 50%. Method D: MeCN held at 0% for 5 min followed by a 7 min ramp to 15% and a 23 min ramp to 35%.

2-[2-[3-(3-(1,7-di-t-butyl-DO2A)propoxy)-2-di(t-butyloxy carbonylmethyl)aminophenoxy]ethoxy]-6-(3-(1,7-di-t-butyl-DO2A)propoxy)-N,N-di(t-butyloxy carbonylmethyl) benzenamine, (2) 640 mg of t-Bu-DO2A (1.6 mmol) and 308 mg K$_2$CO$_3$ (2.24 mmol) were dissolved in 75 mL MeCN. 311 mg compound 1 (.319 mmol) was added and the reaction was stirred at 55 °C with a condenser under nitrogen for 24 hrs, at which point analytical HPLC-MS indicated completion. The crude was filtered and concentrated for HPLC purification. A semi-preparative Polar RP column and Method A (0.1% formic acid additive; retention time = 18 min) were used to purify the product as a pale glass in 30% yield.

2-[2-[3-(3-(1,7-di-ethyl-DO2A)propoxy)-2-di(ethyl oxy carbonylmethyl)aminophenoxy]ethoxy]-6-(3-(1,7-di-ethyl-DO2A)propoxy)-N,N-di(ethyl oxy carbonylmethyl) benzenamine, (2’) 475 mg Et-DO2A (1.4 mmol) and 285 mg K$_2$CO$_3$ (2.1 mmol) were dissolved in 75 mL MeCN. 297 mg compound 1’ (.35 mmol) was added and the reaction was stirred at 40 °C with a condenser under nitrogen for 48 hrs, at which point analytical HPLC-MS indicated completion. The crude was filtered and concentrated for HPLC purification. A semi-preparative Polar RP column and Method A (0.1% formic acid additive; retention time = 13 min) were used to purify the product as a pale glass in 24% yield.
2-[(2-[(3-(3-(1-(1-t-butoxy)-6-((t-butoxycarbonyl)amino)-1-oxohexan-2-yl),4,10-di-t-butyl-DO2A)propoxy)-2-di(1-t-butoxycarbonylmethyl)aminophenoxy ethoxy]-6-(3-(1-(1-t-butoxy)-6-((t-butoxycarbonyl)amino)-1-oxohexan-2-yl),4,10-di-t-butyl-DO2A)propoxy)-N,N-di(t-butoxycarbonylmethyl) benzenamine, (3) 60 mg of compound 2 (.037 mmol) and 51 mg K$_2$CO$_3$ (.37 mmol) were dissolved in 30 mL MeCN. 51 mg of compound 8 (.37 mmol) was added and the reaction was stirred at 40 °C with a condenser under nitrogen for 72 hrs, at which point ESI MS indicated completion. The crude was filtered and concentrated. Silica gel chromatography (gravity) with a gradient of 10→15% MeOH in DCM yielded product as a pale oil in 52% yield. ESI MS m/z observed: 1092.85, calculated: 1092.95 [M + H]$^{2+}$.

2-[(2-[(3-(3-(1-(1-ethoxy)-6-((trifluoroacetyl)amino)-1-oxohexan-2-yl),4,10-di-ethyl-DO2A)propoxy)-2-di(ethoxycarbonylmethyl)aminophenoxy ethoxy]-6-(3-(1-(1-ethoxy)-6-((trifluoroacetyl)amino)-1-oxohexan-2-yl),4,10-di-ethyl-DO2A)propoxy)-N,N-di(ethoxycarbonylmethyl) benzenamine, (3') 117 mg of compound 2' (.084 mmol) and 93 mg K$_2$CO$_3$ (.67 mmol) were dissolved in 25 mL MeCN. 154 mg of compound 8' (.5 mmol) was added and the reaction was stirred at 40 °C with a condenser under nitrogen for 40 hrs, at which point ESI MS indicated completion. The crude was filtered and concentrated. Silica gel chromatography (gravity) with a gradient of 5→12% MeOH in DCM yielded product as a pale oil in 68% yield. ESI MS m/z observed: 948.83, calculated: 948.50 [M + 2H]$^{2+}$.

**Compound 4.** 42 mg of compound 3 (.019 mmol) were dissolved in 10 mL of formic acid and the reaction was stirred at 40 °C with a condenser under nitrogen for 40 hrs, at which analytical HPLC-MS indicated deprotection was complete. Formic acid was removed *in vacuo* and the crude co-evaporated twice with 10 mL DCM to assist acid removal. A semi-preparative Atlantis C18
column and Method D (0.1% formic acid additive; retention time = 16 min) were used to purify the product as a pale glass in 92% yield.

**Compound 4'**. 109 mg of compound 3 (.057 mmol) were dissolved in 16 mL of 1:1 THF/H₂O. 23 mg NaOH (.57 mmol) were added and the reaction was stirred at RT under nitrogen for 24 hrs, at which point analytical HPLC-MS indicated deprotection was complete. The crude was concentrated for HPLC purification. A semi-preparative Atlantis C18 column and Method D (0.1% formic acid additive; retention time = 16 min) were used to purify the product as a pale glass in 58% yield.

**Compound 5**. 47 mg of compound 4' (.033 mmol) were dissolved in 15 mL H₂O and pH was adjusted to 6.5 with dilute NaOH. 45 mg of GdCl₃·6H₂O (.165 mmol) were added and the pH readjusted to 6.5. The reaction was stirred at 40 °C under nitrogen for 18hrs, at which point MALDI MS and analytical HPLC indicated metallation was complete. The reaction was adjusted to pH 9 and filtered to remove excess Gd³⁺ as Gd(OH)₃ and lyophilized. Compound 5 was used without further purification in the next step.

**Compound 9 (1-dye Gd-DOPTA-Fluor)**. Compound 5 (assumed .033 mmol) was dissolved in 5 mL of 100 mM Na₂CO₃ and 4 mL MeCN. 22 mg IR-783-SCN (.0264 mmol) was dissolved in 1 mL DMSO and added to the reaction mixture. The reaction was stirred under nitrogen at RT for 18 hrs covered in tinfoil to prevent photodegradation of the dye. The MeCN was removed in vacuo and the reaction was lyophilized to dryness in the dark for HPLC purification. A semi-preparative X-Bridge C18 column and Method C (1% NH₄OH additive; retention time = 18.5 min) were used to purify the product as a green fluffy solid in 3% yield (over 2 steps) after lyophilization. Analytical HPLC traces (210 and 700 nm λ_abs) of purified compound 9 are shown in Figure 3.3A.
Figure 3.3 – Analytical HPLC traces of purified symmetric Gd-DOPTA-Fluor compounds 9 (A) and 10 (B) obtained using an X-Bridge C18 column and Method E (0.1% NH₄OH additive).
Compound 10 (2-dye Gd-DOPTA-Fluor). Compound 5 (assumed .0043 mmol) was dissolved in 2 mL of 100 mM Na$_2$CO$_3$ and 1.6 mL MeCN. 8.3 mg IR-783-SCN (.0086 mmol) was dissolved in 0.4 mL DMSO and added to the reaction mixture. The reaction was stirred under nitrogen at RT for 18 hrs covered in tinfoil to prevent photodegradation of the dye. The MeCN was removed in vacuo and the reaction was lyophilized to dryness in the dark for HPLC purification. A semi-preparative X-Bridge C18 column and Method B (0.1% NH$_4$OH additive; retention time = 22 min) were used to purify the product as a green fluffy solid in 14% yield (over 2 steps) after lyophilization. Analytical HPLC traces (210 and 700 nm $\lambda_{abs}$) of purified compound 9 are shown in Figure 3.3B.

2-bromo-6-((tert-butoxycarbonyl)amino)hexanoic acid, (7). 1.5 g N$_{\varepsilon}$-Boc-D-lysine (6; 6.09 mmol) were dissolved in 110 mL DCM at chilled to 0°C under nitrogen. 1.5 mL Br$_2$ (30.45 mmol) were added followed by slow addition of isoamyl nitrite over ~ 1 min. The reaction was stirred for 90 min until the starting material had dissolved (indicating reaction of the amine). DCM and Br$_2$ were removed in vacuo and the residue co-evaporated twice with DCM to assist in removal of Br$_2$. Safe handling of the evaporated Br$_2$ was achieved by using an extra cold trap and quenching with concentrated Na$_2$S$_2$O$_3$. After removal of Br$_2$, the crude was taken up in EA and washed 2x with 0.25 M Na$_2$S$_2$O$_3$ followed by 2x with 0.1 M HCl. The organic phase was dried over Na$_2$SO$_4$ and concentrated. Silica gel chromatography with a gradient of 7:3→1:1 hexanes/EA yielded the product as a pale yellow oil in 52% yield. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 6.35 (br s, 1H), 4.52 (dd, $J = 7.2$ Hz, 1H), 3.13 (dd, $J = 6.4$, 0.3, 0.2 Hz, 2H), 2.2-2 (m, 2H), 1.58-1.38 (m, 1H).

2-bromo-6-(2,2,2-trifluoroacetamido)hexanoic acid, (7'). 1g N$_{\varepsilon}$-trifluoroacetyl-D-lysine (6'; 4.13 mmol) and 860 mg KBr were dissolved in 30 mL 1M HBr in a 100 mL beaker. The beaker
was placed in an ice bath and 641 mg NaNO₂ (9.29 mmol) dissolved in 5 mL H₂O was added in portions over 10 min. The reaction was allowed to gradually warm to RT and stirred 18 hrs. An oily residue (crude product) crashed out during this time. The reaction was diluted with H₂O and the product was extracted into DCM in (6×). The combined organic washes were dried over Na₂SO₄ and concentrated to yield pure product as a clear oil in 48% yield. ¹H NMR (500 MHz, CDCl₃) δ 4.19 (dd, J = 8.0, 6.4 Hz, 1H), 3.70 (dd, J = 6.8 Hz, 2H), 2.1-1.9 (m, 2H), 1.5-1.4 (m, 2H).

tert-butyl 2-bromo-6-((tert-butoxycarbonyl)amino)hexanoate, (8). 2.45 mL t-Bu-2,2,2-trichloroacetimidate (13.76 mmol) were added slowly to 1.07 g of compound 7 (3.44 mmol) dissolved in 125 mL CHCl₃ via a drop-funnel. After 18 hrs the crude was concentrated and dry-loaded onto a silica gel column. A gradient of 9:1→5:1 hexanes/EA gave pure product as a pale yellow oil in 83% yield. ¹H NMR (500 MHz, Chloroform-d) δ 4.10 (dd, J = 7.8, 6.8 Hz, 1H), 3.12 (dd, J = 6.7, 6.2 Hz, 3H), 2.14 – 1.86 (m, 2H), 1.48 (s, 9H), 1.46-1.34 (m, 2H), 1.44 (s, 9H).

ethyl 2-bromo-6-(2,2,2-trifluoroacetamido)hexanoate, (8'). 1.5 mL thionyl chloride (20.65 mmol) was added to 75 mL EtOH chilled to 0 °C under nitrogen. After 15 minutes, 605 mg of compound 7' (1.98 mmol) dissolved in 10 mL EtOH was added and the reaction was allowed to gradually warm to RT. After 18 hrs, nitrogen was bubbled through the reaction to remove any HCl gas and the reaction was concentrated. Silica gel chromatography in 7:2 hexanes/EA yielded the product as a pale yellow oil in 71% yield. ¹H NMR (500 MHz, Chloroform-d) δ 4.30 – 4.16 (m, 3H), 3.39 (dd, J = 6.8 Hz, 2H), 1.69 – 1.61 (m, 2H), 1.58 – 1.37 (m, 2H), 1.30 (t, J = 7.1 Hz, 3H).
3.3 Asymmetric Gd-DOPTA-Fluor Agents

Owing in large part to the difficulties in purification of the 1-dye Gd-DOPTA-Fluor agent (compound 9 in Schemes 3.7/3.8), two alternate Gd-DOPTA-Fluor scaffolds were pursued with the goal of eliminating the free amine to alleviate HPLC purification. Both of these compounds were synthesized during completion of the synthesis of both symmetric agents and used several of the same precursors.

3.3.1 Synthesis of 1-dye/1-Gd(III) Probe

The first of the alternate agents is shown in Scheme 3.9 and is obtained in a total of 13 steps, 10 of which are linear. It avoids the free amine by utilizing DO3A rather than DO2A for Gd(III) chelation and decreases total number of synthetic steps by avoiding use of the modified macrocycle arm – the dye is instead conjugated directly to the BAPTA core. Although this CA was expected to have a lower relaxivity than the 2-Gd(III) agents, it would still be Ca\(^{2+}\)-responsive, with only one side of the BAPTA core interacting with the metal center.

The key purification step that suggested the asymmetric core Gd-DOPTA-Fluor agents would be successfully isolated was reaction of the brominated BAPTA core with DO3A. From previous challenges in purification of di-alkylated BAPTA core, it was expected that any residual DO3A would co-elute with the monoalkylated product 2 on normal-phase chromatography. This did in fact occur. However, the solution was simple: make sure all of the DO3A was consumed in the reaction and run a very precise silica plug column. The only way to separate trace DO3A, mono-, and di-alkylated product was to load the crude onto a 1-inch silica plug and perform a very slow ramp in solvent polarity over several hours (1→12% MeOH in DCM). This procedure did not result in complete separation (hence the low yield for this step), but product was pure.
Scheme 3.9 – Synthesis of the asymmetric 1-dye/1-Gd(III) Gd-DOPTA-Fluor analog. This compound bears the IR-783 moiety directly off of the Ca\(^{2+}\)-chelating domain rather than conjugation to a modified macrocycle arm.
After this purification step was established, synthesis of this agent proceeded smoothly until attempts were made to HPLC purify the penultimate intermediate 5. Analytical HPLC-MS suggested that neutral conditions would lead to a well-resolved product peak. However, after the metallated crude was concentrated and injected onto the semi-preparative HPLC, the trace looked completely different and was much dirtier than the analytical had indicated. No product was successful isolated and re-running the concentrated crude on analytical HPLC-MS revealed that rampant degradation had occurred during rotovapping, despite keeping the water bath at near room temperature and not concentrating to complete dryness. With this in mind, intermediate 5 was synthesized again and lyophilized for semi-preparative HPLC purification to avoid degradation. While this worked, the resulting semi-preparative HPLC traces in neutral conditions did not show a well-resolved product peak; instead product streaked off the column over several minutes and was contaminated by other peaks in the chromatogram. A basic injection led to similar results. A final acidic injection led to partial demetallation, but yielded some pure product, which was then reacted with IR-783-SCN to yield the final 1-dye/1-Gd(III) compound 6.

3.3.2 Synthesis of 1-dye/2-Gd(III) Probe

The second asymmetric core Gd-DOPTA-Fluor agent (1-dye/2-Gd(III)) agent is shown in Scheme 3.10 and is obtained in a total of 18 steps, 10 of which are linear. Design of this agent followed naturally from that of the 1-dye/1-Gd(III) compound 6. Compound 6 was not an ideal structure for two main reasons: it had only one Gd(III) and would thus require higher dosage, and it was expected to display poor aggregation properties as a result of eliminating a polar Gd(III) chelate (see section 3.4). The 1-dye/2-Gd(III) compound 6’ is the most elegant of the Gd-DOPTA-Fluor analogs, as it does not contain the unreacted amine. It was not pursued initially, because it
Scheme 3.10 – Synthesis of the asymmetric 1-dye/2-Gd(III) Gd-DOPTA-Fluor analog. Retains conjugation to IR-783 via a modified macrocycle arm. Unlike its symmetric 1-dye counterpart, it does not bear an unreacted 1˚ amine.
must undergo two purification steps that separate macrocyclic intermediates (monoalkylation of the BAPTA core and subsequent alkylation of 2 onto the modified macrocycle 7′), whereas synthesis of the symmetric core agents only requires one macrocyclic separation (see Schemes 3.7/3.8). However, once intermediate 2 was successfully isolated during synthesis of compound 6, it made sense to shoot for compound 6′ as well.

The synthetic route to compound 6′ retained monoalkylation of the BAPTA core by Et-DO3A rather than by the modified macrocycle. This approach was suggested to achieve best separation from TLC analysis. Intermediate 2 was more readily separated from the modified macrocycle 7′ than from Et-DO3A, suggesting that initial monoalkylation using 7′, followed by subsequent alkylation onto Et-DO3A might lead to harder chromatographic separations and lower yields. Predictably, separation of 2 from the modified macrocycle was the most challenging purification step, requiring similar column conditions to purification of 2 discussed previously. However, isolation of 3′ was more challenging, because it ended up co-eluting with unreacted modified macrocycle despite the long, slow ramp. Despite these challenges, enough 3′ was isolated to be carried through the rest of the synthesis. Purification of the deprotected free ligand 4′ proceeded analogously to that of the symmetric core agents using acidic additives. HPLC purification of the final compound 6′ was much easier than that of the 1-dye symmetric analog, upholding the hypothesis that the unreacted amine was the complicating factor. Although the overall yield of 6′ was very low (< 1%), enough compound was isolated to perform relaxometry and other in vitro assays.
3.3.3 Asymmetric Probe Synthetic Methods

All synthetic protocols and instrumentation followed those outlined in Section 3.2.2. Synthetic preparation of Et-DO3A, Et-DO2A, IR-783-SCN, and the brominated BAPTA core all directly followed literature precedent. Synthesis of the trifluoroacetyl-protected α-bromo ester arm is outlined in Section 3.2.2 (compound 8 in Scheme 3.8).

2-{2-[3-{3-(Tris-ethyl-DO3A)propoxy]-2-di(ethoxycarbonylmethyl)aminophenoxy] ethoxy}-6-(3-bromopropoxy)-N,N-di(ethoxycarbonylmethyl) benzenamine, (2). 802 mg of compound 1 (.93 mmol) and 308 mg K₂CO₃ (2.23 mmol) were dissolved in 1200 mL MeCN. 488 mg of Et-DO3A (1.11 mmol) was added and the reaction was stirred at 60 °C with a condenser under nitrogen for 40 hrs, at which point ESI MS indicated consumption of the Et-DO3A. The crude was filtered and concentrated. Silica gel chromatography (gravity) with a very slow gradient of 2.5→5% →7.5% MeOH in DCM yielded product as a pale oil in 43% yield. ESI MS m/z observed: 1211.66, calculated: 1211.53 [M + H]⁺.

2-{2-[3-{3-(Tris-ethyl-DO3A)propoxy]-2-di(ethoxycarbonylmethyl)aminophenoxy] ethoxy}-6-(3-azidopropoxy)-N,N-di(ethoxycarbonylmethyl) benzenamine, (3). 490 mg of compound 2 (.404 mmol) were dissolved in 50 mL DMF. 525 mg NaN₃ (8.08 mmol) were added and the reaction was stirred at 70 °C with a condenser under nitrogen for 18 hrs, at which point ESI MS indicated complete conversion to the azide. The reaction was filtered and the DMF removed in vacuo. The crude residue was taken up in DCM/H₂O and extracted into DCM 3x. The combined organic phases were dried over Na₂SO₄ and concentrated. ESI MS m/z observed: 1174.76, calculated: 1174.62 [M + H]⁺.
2-[2-{3-(3-(1-(ethoxy)-6-((trifluoroacetyl)amino)-1-oxohexan-2-yl),4,10-di-ethyl-DO2A)propoxy)-2-di(ethoxycarbonylmethyl)aminophenoxy] ethoxy}-6-(3-(Tris-ethyl-DO3A)propoxy)-N,N-di(ethoxycarbonylmethyl) benzenamine, (3'). 352 mg of compound 3 (.29 mmol), 260 mg of compound 7' (.435 mmol), and 120 mg of K$_2$CO$_3$ (.87 mmol) were dissolved in 50 mL MeCN. The reaction was stirred at 70 °C with a condenser under nitrogen for 48 hrs, at which point ESI MS indicated compound 2 was largely consumed and the reaction was filtered and concentrated. Silica gel chromatography (gravity) with a very slow gradient of 2.5→5%→7.5%→10%→15% MeOH in DCM yielded product as a pale oil in 7% yield. ESI MS m/z observed: 865.12, calculated: 864.97 [M + 2H]$^{2+}$.

**Compound 4.** Compound 3 (assumed .4 mmol) was dissolved in 50 mL THF. 25 mL 1M NaOH were added and the biphasic reaction mixture was stirred at 35 °C under nitrogen for 24 hrs, at which point MALDI MS indicated completion of deprotection. THF was removed in vacuo and the residual aqueous mixture adjusted to pH 6.5 with 4M HCl. This was carried through without further purification to synthesis of penultimate intermediate compound 5. ESI MS m/z observed: 978.65, calculated: 978.44 [M + H]$^+$.  

**Compound 4'.** 35 mg of compound 3 (.02 mmol) were dissolved in 20 mL THF. 10 mL 1M NaOH were added and the biphasic reaction mixture was stirred at 35 °C under nitrogen for 18 hrs, at which point analytical HPLC-MS indicated deprotection was complete. THF was removed in vacuo and the aqueous crude was neutralized then lyophilized down for HPLC purification. A semi-preparative Polar RP column and Method E (0.1% formic acid additive; retention time = 14 min) were used to purify the partially demetallated product as a pale powder after lyophilization in approximately 41% yield.
Compound 5. 450 mg GdCl\textsubscript{3}·H\textsubscript{2}O was added to the aqueous mixture containing compound 4. pH was readjusted to 6.5 with dilute NaOH and approximately 200 mg Pd/C was added. The flask was sealed with a septum and parafilm and flushed with H\textsubscript{2}. The metallation/catalytic hydrogenolysis reaction was stirred 18 hrs, at which point analytical HPLC indicated product had been formed. The reaction was adjusted to pH 8.5 with NaOH to precipitate residual Gd\textsuperscript{3+} as Gd(OH)\textsubscript{3} and filtered and lyophilized for HPLC purification. A semi-preparative Polar RP column and Method D (0.1% formic acid additive; retention time = 18 min) were used to purify the partially demetallated product as a pale powder after lyophilization in approximately 18% yield.

Compound 5’. 11 mg of compound 4’ (.0081 mmol) were dissolved in 15 mL H\textsubscript{2}O and pH was adjusted to 6.5 with dilute NaOH. 10 mg of GdCl\textsubscript{3}·6H\textsubscript{2}O (.024 mmol) were added and the pH readjusted to 6.5. The reaction was stirred at RT under nitrogen for 18hrs, at which point MALDI MS and analytical HPLC indicated metallation was complete. The reaction was adjusted to pH 8.5 and filtered to remove excess Gd\textsuperscript{3+} as Gd(OH)\textsubscript{3} and lyophilized. Compound 5’ was used without further purification in the next step.

Compound 6. 161 mg of Compound 5 (max .07 mmol) was dissolved in 5 mL of 100 mM Na\textsubscript{2}CO\textsubscript{3} and 4 mL MeCN. 15 mg IR-783-SCN (.018 mmol) was dissolved in 1 mL DMSO and added to the reaction mixture. The reaction was stirred under nitrogen at RT for 18 hrs covered in tinfoil to prevent photodegradation of the dye. The MeCN was removed \textit{in vacuo} and the reaction was lyophilized to dryness in the dark for HPLC purification. A semi-preparative X-Bridge C18 column and Method C (0.1% NH\textsubscript{4}OH additive; retention time = 18 min) were used to purify the product as a green fluffy solid in 4% yield after lyophilization. Analytical HPLC traces (210 and 700 nm \(\lambda_{abs}\)) of purified compound 6 are shown in Figure 3.4A.
Figure 3.4 – Analytical HPLC traces of purified asymmetric Gd-DOPTA-Fluor compounds 6 (A) and 6’ (B) obtained using an X-Bridge C18 column and Method E (0.1% NH₄OH additive).
**Compound 6'.** Compound 5' (assumed .0081 mmol) was dissolved in 2.5 mL of 100 mM Na$_2$CO$_3$ and 1.6 mL MeCN. 11 mg IR-783-SCN (.012 mmol) was dissolved in 0.5 mL DMSO and added to the reaction mixture. The reaction was stirred under nitrogen at RT for 24 hrs covered in tinfoil to prevent photodegradation of the dye. The MeCN was removed in vacuo and the reaction was lyophilized to dryness in the dark for HPLC purification. A semi-preparative X-Bridge C18 column and Method C (0.1% NH$_4$OH additive; retention time = 17 min) were used to purify the product as a green fluffy solid in 17% yield (over 2 steps) after lyophilization. Analytical HPLC traces (210 and 700 nm $\lambda_{abs}$) of purified compound 6' are shown in Figure 3.3B.

1-(1-(ethoxy)-6-((trifluoroacetyl)amino)-1-oxohexan-2-yl),4,10-di-ethyl-DO2A (7'). 1.08g of Et-DO2A (3.24 mmol) and 750 mh K$_2$CO$_3$ (5.4 mmol) were dissolved in 150 mL MeCN. 549 mg of the trifluoroacetyl-N$_e$-L-lysine $\alpha$-bromo ester arm (1.8 mmol) were added and the reaction was stirred under nitrogen at RT for 40 hrs. The reaction was filtered and concentrated. Silica gel chromatography in 10% MeOH/EA yielded the product as a light brown oil in 43% yield. ESI MS m/z observed: 598.30, calculated: 598.33 [M + H]$^+$.

**3.4 Relaxometry of Gd-DOPTA-Fluor Agents**

The 2-dye/2-Gd(III) compound 10 (Scheme 3.8) was the first Gd-DOPTA-Fluor agent successfully isolated and characterized. In order to verify that dye conjugation did not affect Ca$^{2+}$-binding, $r_1 \pm$ Ca$^{2+}$ was measured. Relaxometry was performed on a Bruker mq60 NMR analyzer operating at 1.41T and 37 °C. $T_1$ (s$^{-1}$) was measured for several different [CA] and the inverse was plotted against Gd(III) concentration (mM) and fit via linear regression. The slope of the line corresponds to $r_1$ by

$$\frac{1}{T_{1,obs}} = \frac{1}{T_{1,d}} + r_1[Gd(III)]$$  (3.1)
Accurate [Gd(III)] was determined by inductively coupled plasma mass spectrometry (ICP-MS).

A serial dilution of compound 10 dissolved in 100 mM HEPES containing 100 mM KCl at pH 7.4 yielded 5 samples for relaxometry. An aliquot of each sample was taken for ICP to determine [Gd(III)] before change in volume due to Ca\(^{2+}\) addition. After measuring Ca\(^{2+}\)-free \(T_1\) of each sample, a small volume of concentrated CaCl\(_2\) solution was added and the samples were thoroughly vortexed to insure proper mixing. However, it was immediately visibly obvious that Ca\(^{2+}\)-addition had caused the 2-dye/2-Gd(III) Gd-DOPTA-Fluor agent to precipitate out of solution (Figure 3.5). The \(T_1\) values measured after Ca\(^{2+}\) addition reflected this, as they increased with time as the precipitate settled and eventually approached \(T_1\) value measured for buffer alone. As the \(T_1\) values varied with time, \(r_1, Ca^{2+}\) was not calculated. In an attempt to resolubilize the precipitate, DMSO was added to the samples (up to 15%) and \(T_1\) was remeasured. DMSO addition had minimal impact on \(T_1\) and the agent was still visibly precipitated. The precipitate only dissolved upon addition of 40% MeCN and when this mixture was investigated on analytical HPLC-MS, no compound 10 was detected.

A literature search suggested that the most likely cause of CA precipitation was aggregation of the dye via \(\pi-\pi\) stacking interactions.\(^{17}\) In the absence of Ca\(^{2+}\), the negative charges on the BAPTA domain were hydrophilic enough to prevent formation of large, insoluble aggregates. However, once Ca\(^{2+}\) chelation occurred, the agent lost enough charge that larger aggregates rapidly formed and precipitated out of solution. Compound 10 would be more prone to this than the 1-dye/2-Gd(III) agents because it has an extra dye moiety to participate in stacking interactions. This was true for each sample used in relaxometry of compound 10 and spanned a [CA] gradient that covered two orders of magnitude. Although most highly conjugated aromatic fluorophores will
Figure 3.5 – Aggregation of 2-dye asymmetric Gd-DOPTA-Fluor compound 10 in response to Ca$^{2+}$ at two different [CA].

HEPES buffer; pH 7.4; 10mM Ca$^{2+}$
aggregate at high enough concentrations (quenching fluorescence), this is not as problematic for optical probes, as their dosage is often below the critical micelle concentration of the dye, or aggregation can be alleviated by doping with very small (typically < 1%) amounts of biocompatible organics that break up aggregates (e.g. DMSO or a surfactant). Given the high dosage of MR probes necessary for MR detection it was clear that Gd-DOPTA-Fluor agents would likely need a surfactant, DMSO, or a combination of both methods to remain in solution after Ca\(^{2+}\) chelation. Necessity of a surfactant was further suggested from failure of DMSO to resolubilize the aggregates observed in relaxometry. Thus, once the symmetric 1-dye/2-Gd(III) compound 9 was isolated, it was subjected to a surfactant screen prior to measuring relaxivity.

In order to determine which surfactant/DMSO strategy led to minimal dye aggregation, fluorimetry was used to test several different formulations with minimal consumption of CA. Emission spectra (\(\lambda_{\text{exc}} = 760\) nm, recorded from 776 to 825 nm) ± Ca\(^{2+}\) were measured for compound 9 on a Hitachi F4500 Fluorimeter. Decrease in the intensity of \(\lambda_{\text{em, max}}\) and blue-shifting of its value are both indicative of aggregation. In the first experiment, several different biologically-compatible surfactants were screened to determine which one led to the smallest \(\lambda_{\text{em, max}}\) shift. In this experiment, the surfactants were dosed at fixed concentrations which were not expected to kill cells, based on literature precedent. The surfactants investigated were dosed as follows: 0.1% Pluronic F-127, 0.1% Kolliphor EL, 4 mM cholate, 0.1% Tween-80/1% DMSO, and 1% Tween-80/1% DMSO. Although Tween is toxic to cells, these conditions were included because they have been shown to prevent aggregation of IR-783 analogs. All test solutions were made up in 100 mM HEPES buffer with 100 mM KCl at pH 7.4. For each surfactant condition, the final CA solution was approximately 1 \(\mu\)M and had two distinct formulations: dilution of an
aliquot of CA in HEPES buffer into the surfactant buffer and dilution of an aliquot of CA in DMSO into the surfactant buffer (final [DMSO] = 1%; the Tween conditions also had 1% DMSO but differed in whether the CA aliquot was in DMSO or buffer). The DMSO aliquot-derived formulations were included to determine whether dispersing the CA from a non-aggregated organic solution led to decreased aggregation. A HEPES only formulation ± DMSO was also included as a point of reference (since 2-dye/2-Gd(III) Gd-DOPTA-Fluor was known to precipitate in HEPES after Ca^{2+} addition). The emission spectra of each formulation ± Ca^{2+} obtained in the first screening experiment are shown in Figure 3. In Figure 3, for each surfactant, “D” indicates that the CA was aliquoted from DMSO and “+/−” indicate presence/absence of 1 mM Ca^{2+}. For all surfactants, the DMSO aliquot was redshifted independent of Ca^{2+} addition, indicating that aliquoting from DMSO leads to better dispersion of CA and decreases aggregation. Unsurprisingly, the HEPES formulations had the lowest fluorescence intensity and the biggest decrease in fluorescence intensity after Ca^{2+} addition, corresponding to the most aggregation. Unfortunately, the only surfactant formulation that did not show a drop in fluorescence intensity with Ca^{2+} addition was 1% Tween-80, which is cytotoxic.

However, cholate and Kolliphor EL can both be dosed at concentrations higher than 4mM and 0.1% (respectively), so a second fluorescence screening experiment was performed to determine if increasing the concentration of these surfactants would decrease aggregation. In this experiment, all surfactant formulations started from a DMSO CA stock, such that the final [DMSO] = 1% and the final [CA] was ~ 1 µM. Tween-80 was also screened to see how the two biocompatible surfactants compared to it. The results of the second screening experiment are shown in Figure 3.7. Although Tween-80 and Kolliphor EL out-performed cholate (as seen by
Figure 3.6 – Effect of surfactant formulation on aggregation of the 1-dye symmetric compound 9. Blue-shifts and decrease in fluorescence intensity are indicative of increased aggregation. All plots have the same legend. +/− indicates 1 mM/0 mM Ca$^{2+}$. D indicates that the CA was aliquoted initially from DMSO (to a final [DMSO] = 1%).
Figure 3.7 – Effect of varying surfactant concentration on aggregation of the 1-dye symmetric compound 9. + indicates 1 mM Ca$_{2}^{+}$. All formulations started from a DMSO aliquot of the CA to maximize dispersion in aqueous media.
greater fluorescence intensity), the 10 mM cholate formulation did not show a change in emission after addition of Ca\(^{2+}\).

Since 10 mM cholate is the most biocompatible of the surfactant conditions tested in the second experiment, relaxometry of 1-dye/2-Gd(III) compound 9 was measured in 10 mM cholate in HEPES/KCl buffer, with 1% DMSO from the initial CA aliquot. Upon addition of Ca\(^{2+}\) to the first relaxometry sample, the CA again precipitated out completely and \(T_1\) approached that of buffer alone. In an attempt to mitigate this, concentrated Kolliphor EL was added to the second sample to a final [Kolliphor EL] = 1%, but the CA still crashed out after Ca\(^{2+}\) addition. The third relaxometry sample was brought to 1% Tween-80 to no avail. As a last-ditch-effort, the fourth and fifth relaxometry samples were subjected to serial dilutions to create new relaxometry samples formulated in 5% Tween-80 and 5% Kolliphor EL (still in a base buffer of HEPES/KCl with 1% final [DMSO]). Both of these surfactant formulations led to a decrease in \(T_1\) after Ca\(^{2+}\) addition! The \(\Delta T_1\) was greater for the Tween-80 samples due to slow aggregation of the CA in the 5% Kolliphor EL samples (by 4 hrs the Kolliphor samples were completely precipitated). However, 5% Tween-80 led to stabilization of the Ca\(^{2+}\)-bound CA for >24 hours, determined by visual inspection and \(T_1\) measurement.

The successful Tween-80 conditions (5% Tween-80 in HEPES/KCl buffer with CA aliquoted from DMSO to a final [DMSO] = 1%) were then used to measure relaxivity of the 2-dye/2-Gd(III) compound 9, and the 1-dye/1-Gd(III) asymmetric agent 6. Compound 9 successfully showed a decrease in \(T_1\) and did not precipitate out after Ca\(^{2+}\) addition. Compound 6 did not display any initial \(\Delta T_1\) in response to Ca\(^{2+}\), but after several hours it began to precipitate, and had crashed out completely by 18 hours. Most-likely it was initially “unresponsive” to Ca\(^{2+}\),
because the aggregation-induced decrease in $r_1$ was balanced by the $q$-modulated increase in $r_1$ from Ca$^{2+}$ chelation. Figure 3.8 shows the $r_1$ values measured for compounds 9, 10, and 6, as well as their structures. Although they required a highly cytotoxic amount of Tween-80 to prevent formation of insoluble aggregates in the presence of Ca$^{2+}$, this should not pose a problem for evaluation of the Gd-DOPTA-Fluor agents in tissue culture and in vivo, since the agents are all stable in cell media (DMEM + 10% FBS) from a DMSO aliquot (final [DMSO] = 1%) and DMEM contains > 2.5 mM Ca$^{2+}$. Unfortunately, cell media was not able to be used in relaxometry because it is very hard to remove its Ca$^{2+}$ and Zn$^{2+}$, both of which would prevent measurement of $r_{1,\text{off}}$.

All three of the Gd-DOPTA-Fluor agents in Figure 3.8 had higher than expected $r_1$ values for small molecule CAs. Unfortunately this corresponded to a much small Ca$^{2+}$-dependent $\Delta r_1$ (35% for the symmetric 1-dye/2-Gd(III) compound 9 and 15% for the 2-dye/2-Gd(III) compound 10). The high measured $r_1$ values are likely the result of a $\tau_R$-modulated boost due to aggregation of the dye. This is supported by the decrease in $r_1$ observed in conditions that did not lead to precipitation (e.g. decreasing aggregation led to smaller molecular weight aggregates which were not $\tau_R$-optimized).

### 3.5 Outlook and Future Work

Four new Gd-DOPTA-Fluor agents were successfully synthesized and subjected to preliminary relaxometric characterization. The agents need to be evaluated in tissue culture to determine whether conjugation to IR-783 has led to improved cellular uptake. The agents display a decreased $\Delta r_1$ in response to Ca$^{2+}$ than the original Gd-DOPTA agent, most likely due to aggregation-induced boost in contribution of $\tau_R$ to $r_1$. However, it is unknown how the intracellular environment will modulate aggregation of the agents. In addition, contributions of $\tau_R$ to $r_1$ fall off
Figure 3.8 – Ionic relaxivity values of Compounds 9, 10, and 6 and corresponding $\Delta r_1$. The more aggregation-prone species (10 and 6) have higher $r_1$ values most likely due greater effective molecular weight and boost in $\tau_R$ contributions to inner sphere relaxation.
dramatically with increasing magnetic field strength. Performing relaxometry at 9.4T (the field strength of \textit{in vivo} MR imaging experiments) would reveal whether a larger $\Delta r_1$ is observed when contributions of $\tau_R$ to $r_1$ are less significant and would be more indicative of the potential for the agent have a detectable $\Delta r_1 \textit{ in vivo}$. Assuming the Gd-DOPTA-Fluor agents display a greater magnitude $\Delta r_1$ at 9.4T and have improved cellular uptake over Gd-DOPTA-Et, the agent with best performance in tissue culture will be evaluated \textit{in vivo} in the modified mouse seizure model outlined in Section 2.3.3.
Chapter 4: References
4.1 Chapter 1 References

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4.2 Chapter 2 References


4.3 Chapter 3 References


Appendix: Bioorthogonal Methods for Detection of Cancer Biomarkers by MRI
A1 Bioorthogonal Chemistry and Motivation for its use in MRI

Cells can often be characterized by specific surface markers, which range from hormone receptors to particular sugars in the extra cellular matrix. These markers are useful for distinguishing between healthy cells and diseased cells and can also be useful to image a specific tissue or organ. In addition, they are often also able to shed light on the progression of the particular disease (as in triple hormone receptor status of breast cancers). Thus, molecular imaging (MI) often relies on conjugating imaging moieties to probes that target cell surface markers of choice.

In order to reduce background signal and the leeching of imaging agents into surrounding media and tissues, MI systems often utilizes bioorthogonal labeling strategies. These methods rely on reactive groups that are orthogonal to endogenous functional groups, making it possible to noninvasively yet selectively target and image a population of cells or organ of choice. Bioorthogonal labeling relies on pre-incubation with a targeted probe bearing a bioorthogonal reactive group. After sufficient time has passed to clear unbound probe from the system, an imaging modality with complimentary bioorthogonal functionality is introduced. Once again, excess and/or unreacted imaging agent is allowed to clear from the system, at which point the sample is imaged. Because imaging agent can only accumulate at the target site, background signal is significantly reduced, allowing for enhanced contrast.

The bioorthogonal approach could also allow for decreased background signal from nonspecific binding as compared to incubation with contrast agents bearing a targeting moiety, since the components of the bioorthogonal system are smaller and excreted more rapidly. As a result of enhanced clearance of unbound compound, a bioorthogonal labeling strategy requires less
time for clearance of nonspecifically bound contrast agent, decreasing the probability of bound agents dissociating from the target and maximizing MR image contrast at the target.

**A2 FR- and MMP-targeted Probes**

The ultimate goal of most of the work carried out on this project was to non-invasively detect matric metalloproteinases (MMPs) and folate receptor (FR) *in vivo* using a bioorthogonal targeting group/CA pair. MMPs and FR are established cancer biomarkers and are common targets of imaging agents in the literature. A previous graduate student (Dr. Preeti Sukerkar) designed the original probes and chose the bioorthogonal reaction between norbornene and tetrazine (Figure 4.1A). Norbornene is much more synthetically stable, so it was used to modify the targeted probes, while tetrazine was conjugated to a Gd(III) chelate at the last step of synthesis under mild conditions to avoid its degradation. The targeted probes utilized folate to target FR and a biphenyl sulfonamide MMP inhibitor to target MMPs. The original norbornene-probe and tetrazine-CA structures proposed by Preeti are shown in Figure 4.1B.

These targeted norbornene-probes are, unfortunately, not water stable. In the ideal bioorthogonal labeling strategy discussed above, the bioorthogonal probes should be water stable for fast clearance. In addition, because the probes would be detected by MR imaging, they needed to be dosed at very high concentrations (enough to overcome the detection limit and pass biological clearance mechanisms) not compatible with hydrophobic species. Thus, the synthetic goal of this project became to develop water-soluble norbornene probes. A side goal was to develop a multiplexed tetrazine-CA bearing multiple Gd(III) chelates for higher Gd(III) payload delivery to the target sites (see Scheme 4.1).
Figure 4.1 – Bioorthogonal labeling with MRI CAs. A) Reaction between norbornene and tetrazine is irreversible due to release of N$_2$ gas. B) Original probe structures designed by Dr. Preeti Sukkerkar.
The first attempt to achieve water solubility of the norbornene-probes added at triazole in the linker between the norbornene and the targeting group (Figure 4.2A). It is not surprising that neither of these structures were anywhere near water soluble. The second modification to increase hydrophilicity involved incorporation of a PEG linker (modified tri-, tetra-, and pentaethylene glycol) between the triazole and the probe (Figure 4.2B). These structures utilized PEG linkers that were modified to bear an azide for clicking onto the alkyne-modified norbornene. They were also not water soluble or soluble in water with minimal DMSO. The third and final attempt to improve water solubility of the norbornene probes was to remove the triazole and utilize an amide linker between the PEG and the norbornene (Figure 4.2C). The folate pentaethylene glycol norbornene-probe structure was water soluble, but the biphenyl sulfonamide norbornene-probes were not. Although synthetic progress was made towards the multiplex tetrazine-CA (Scheme 4.1), the penultimate intermediate was very prone to photodegradation and polymerization and no click conditions were found that did not also reduce the tetrazine in the final step.

Right after obtaining the water-soluble PEG folate norbornene-probe, a previous student in the lab (Dr. Luke Vistain) did some very elegant work to elucidate the MR detection limit of biomarkers by small molecule Gd(III) agents. He conclusively proved that $10^6$ surface-expressed proteins per cell is at least two orders below the MR detection limit of small molecule Gd(III) CAs. Fortunately for him, using a nanoparticle platform solved this problem and led to a publication. Unfortunately for the bioorthogonal project as designed, that was the nail in the coffin. MMPs are expressed much lower than $10^6$ copies per cell and are secreted, further decreasing their effective expression at the target site. Although FR can achieve close to $10^6$ copies per cell expression, the receptor internalizes. This means a large fraction of bound norbornene-probe will be intracellular
Figure 4.2 – Three new generations of norbornene-probes were to developed with goal to achieving water-solubility.

Scheme 4.1 – Synthetic efforts toward a multiplex tetrazine CA. The tetrazine was not compatible with reducing metals used in click chemistry.
and, unfortunately, the tetrazine-CA is not very cell-permeable (Preeti did an uptake study and found that it is taken up by cells as well as the clinical blood-pool agent Gd-DOTA). For these reasons the norbornene-tetrazine bioorthogonal approach was abandoned.

**A3 Sialic Acid-targeted Nanoparticle Probes**

As discussed in Section 1.4.4, nanoparticles can increase delivery of Gd(III) to target sites by several orders of magnitude. The next approach to achieving bioorthogonal labeling of cancer biomarkers by MRI thus proposed to employ spherical nucleic acid gold nanoparticles bearing Gd(III) chelates to image cell-surface sialylation. Changes in cell-surface sialylation patterns have been correlated to many disease pathways, including cancer progression and metastasis. They are thus widely studied in the literature and are an attractive target. In addition, cancer cells can achieve very high levels of sialic acid expression in the extracellular matrix which should be detectable by nanoparticle Gd(III) CAs. The bioorthogonal nanoplatform proposed here utilized the reaction of dibenzylcyclooctyne (DBCO) with azides. The nanoparticle would bear DBCO moieties for reaction with an azide on an unnatural sugar (azido N-acylmannosamine) incorporated into the extracellular matrix. The azido sugar is similar in structure to the native biological precursor to sialic acid and the glycosyltransferase enzymes that incorporate it into cell-surface glycans are promiscuous enough to incorporate it instead of the natural sugar. The azido sugar is simply added to tissue culture media or animal feed and can then be imaged bioorthogonally.

Design of the nanoplatform is shown in Figure 4.3. 14 nm spherical gold nanoparticles were synthesized and characterized by TEM. Then, commercially available DBCO NHS-ester was peptide coupled to commercially available thiolated PolyT DNA bearing an amine. The modified
Figure 4.3 – Nanoplatform for bioorthogonal detection of cell-surface sialylation with increased detection limit due to large Gd(III) payload achieved in nanoparticle systems.
DNA was purified by size exclusion and characterized by MALDI MS. A Gd(III) chelate bearing a dithiolane for binding to the gold nanoparticle surface was synthesized (Scheme 4.2).

A3.1 Future Work

Once the DBCO-DNA and dithiolane-CA are loaded onto the nanoparticles, this nanoplatform can be readily characterized in tissue culture. There are many established cell lines with a spectrum of sialylation levels. In addition, there are many commercially available DBCO-functionalized fluorophores that will assist in quantifying how well the nanoplatform binds the azido sugar in tissue culture by flow cytometry.

A4 Appendix References

Scheme 4.2 – Synthesis of a dithiolane-modified Gd(III) chelate for adsorption onto the surface of the gold nanoparticle bioorthogonal platform and ESI MS of the purified product.