MOLECULAR IMAGING OF BREAST CANCER

USING PARACEST MRI

by

BYUNGHEE YOO

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Thesis Advisor: Prof. Mark D. Pagel, Ph.D.

Department of Biomedical Engineering

CASE WESTERN RESERVE UNIVERSITY

August, 2007
We hereby approve the dissertation of

______________________
BYUNGHEE YOO

candidate for the Doctor of Philosophy degree *.

(signed) Mark D. Pagel, Ph.D.

(chair of the committee)

Raymon F. Muzic, Ph.D.

Suneel S. Apte, Ph.D.

Xin Yu, Sc.D.

(date) June 15, 2007

*We also certify that written approval has been obtained for any proprietary material contained therein.
DEDICATION

To my family and my mentors
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MRI is an excellent diagnostic method for detecting breast cancer, but suffers from an inability to identify cancerous lesions from non-cancerous lesions. MRI contrast agents that are responsive to biomarkers that are only associated with breast cancer lesions and cancer therapies may improve the specificity of breast cancer diagnoses with MRI. In order to develop these new responsive MRI contrast agents, new synthesis methodologies were developed to generate peptide-DOTA conjugates with high purity and high yield. These peptide-DOTA MRI contrast agents can be detected via PARAMagnetic Chemical Exchange Saturation Transfer (PARACEST). A peptide-DOTA PARACEST agent was developed that detects caspase-3, which is an important biomarker of tumor cell apoptosis following cancer therapy. PARACEST agents that can be selectively detected were also developed that can measure tumor vascular permeability, which is associated with tumor angiogenesis. Dynamic Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI) was performed using PARACEST contrast agents and the results were compared with conventional DCE-MRI. These responsive and selectively detectable PARACEST MRI contrast agents constitute a new platform technology for molecular imaging of breast cancer.
To address the diagnosis of angiogenesis and apoptosis, this thesis has 3 aims:

a) Development of new synthetic pathways for PARACEST MR contrast agents

b) Evaluation of enzyme responsive PARACEST MR contrast agents

c) Application of PARACEST MR contrast agents to DCE-MRI
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List of Abbreviations

Ac₂O: acetic anhydride
ACN: acetonitrile
AMC: aminomethyl coumarin
BaO: barium oxide
CCl₄: carbon tetrachloride
CHAPS: 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid
DCM: dichloromethane
DIEA: N,N-diisopropylethylamine
DMF: N,N-dimethylformamide
DO3A-tBu: 1,4,7,10-tetraazacyclododecane-\(N,N',N''\)-tri(tert-butylacetate)
EDTA: ethylenediamine tetraacetate
Fmoc: fluorenlymethoxy carbonyl
HBTU: 2-(1\(H\)-benzotriazole-1-yl)-1,1,3,3-tetramethuronium hexafluorophosphate,
HOBT: N-hydroxybenzotriazole
MALDI: Matrix-assisted laser desorption/ionization
MSME: multi slice multi echo
NMP: N-methylpyrrolidone
P₂O₅: phosphorous pentoxide
PIPS: Piperazine-1,4-bis(2-ethanesulfonic acid)
RARE: rapid acquisition with relaxation enhancement
TEA: triethylamine
Z: carbobenzoxy carbonyl (CBZ)
In Chapter I. Introduction, two important physiological phenomena in breast cancer, apoptosis and angiogenesis, are described. Two molecular biomarkers, caspases for apoptosis and matrix metalloproteinase’s for angiogenesis, are described particularly in the context of molecular imaging detection methods. In Chapter II. Responsive MR contrast agents, the responsive contrast agents in MR imaging are reviewed based on the response mechanisms, such as tumbling time, water accessibility and water exchange rates. Detecting enzyme activity has wide applications and is a hot issue in molecular imaging research. In Chapter III. Solution phase synthesis of peptidyl contrast agents, a new facile synthetic methodology for creating peptidyl DOTA using an aminoDOTA core compound is described. The solution phase synthesis methodology has limitations for the introduction of diverse sequences of peptides on a DOTA moiety. To overcome this limitation, in Chapter IV. Solid phase synthesis of peptidyl contrast agents, a solid phase synthetic approach was investigated. The solid phase synthetic method has been applied
for the synthesis of diverse peptidyl DOTA structures for research presented in this dissertation, and for other molecular imaging research.

In Chapter V. Detection of enzyme activity based on PARACESET effect, the newly synthesized caspase-3 targeting peptidyl MR contrast agent was evaluated for the physico/chemical properties. The *in vitro* enzyme activity detection was demonstrated using caspase-3 and the newly synthesized peptidyl DOTA MR contrast agent. Kinetics studies were conducted by measuring PARACEST effects and the PARACEST kinetics results were compared with the results from fluorescence enzyme kinetics studies.

In Chapter VI. Review of DCE-MRI contrast agents, the contrast agents used for DCE-MRI were reviewed according to the criteria of molecular weight. The conventional DCE-MRI method was implemented and tested to show the utility of this technique to study tumor angiogenesis. PARACEST DCE-MRI is new concept of DCE-MRI in many aspects. The PARACEST DCE-MRI technique was studied and the results were compared with conventional T₁ DCE-MRI. The results were summarized in Chapter VII. PARACEST DCE-MRI.

In Chapter VIII. Future Research, the research directions in the future and tentative projects are suggested.
Chapter I. Introduction
1. **Breast cancer and molecular MR imaging**

   **A. Breast cancer and biomarkers**

Breast cancer continues to be a major health problem worldwide despite recent advances in its diagnosis and treatment.\(^1\) Breast cancer is by far the most common cancer of women, comprising 23% of all female cancers, and there were an estimated 1.15 million new cases in 2002.\(^2\) It ranks second overall when both sexes are considered. More than half of all cases occur in industrialized countries - about 361,000 in Europe (27.3% of cancers in women) and 230,000 in North America (31.3%).\(^3\) Estimates suggest that one in nine women will develop breast cancer in her lifetime, about one-third of who will die from it.\(^4\)

Decreased apoptosis and increased angiogenesis may play important roles in the biological aggressiveness of breast cancer. Apoptotic pathways and angiogenic status are controlled by a number of regulators, including inducers and inhibitors relevant to the pathogenesis of breast cancer. Apoptosis and angiogenesis are considered as novel prognostic indicators in breast cancer, and they may have predictive value for the response to anticancer treatments.\(^5\)

Apoptosis is also known as “programmed cell death” and attracts much interest in cancer research.\(^6\) Caspases play important roles in apoptosis. Caspases are usually synthesized
as inactive proenzymes that are activated by many types of stimuli. The most intensively studied apoptotic caspase is caspase-3, previously called CPP32, Yama or Apopain. Caspase-3 normally exists in the cytosolic fraction of cells as an inactive precursor that is activated proteolitically when cells are signaled to undergo apoptosis. 

In the apoptosis cascade, two routes have been identified to activate caspase-3 by caspase-8. In one route, caspase-8 directly processes procaspase-3 and caspase-3 cleaves various cellular proteins. In another route, caspase-8 cleaves Bid, a pro-apoptotic member of the Bcl-2 family, which translocates to mitochondria to release cytochrome C into the cytosol. The cytochrome C then activates caspase-9 together with Apaf-1, and caspase-9 in turn activates caspase-3.

Angiogenesis, the formation of new blood vessels, is a crucial process during development. Angiogenesis is also a necessary step for tumor progression, as tumors cease to grow beyond 1.5 ~ 2 mm in diameter without the nutrient and oxygen supply from new blood vessels. This concept has yielded a myriad of strategies to inhibit tumor growth by angiogenesis modulators. These include protease inhibitors, inhibitors of growth factors and growth factor receptors, integrin inhibitors, inhibitors of signaling cascades and many other agents with undefined mechanisms. MMP’s, especially the gelatinases (MMP-2 and MMP-9), are critically involved in angiogenesis in vitro and
in vivo.\textsuperscript{28,29} The exact mechanisms of how the gelatinases contribute to angiogenesis still remain obscure and may include multiple pathways.\textsuperscript{30} The most direct evidence for gelatinases in angiogenesis comes from the studies of the initial steps in tumor angiogenesis.

In a model of carcinogenesis of pancreatic islets in RIP1-Tag2 transgenic mice, MMP-2 and MMP-9 were found to be upregulated in angiogenic lesions. The upregulation of the gelatinases resulted in the release of bioactive VEGF, which is a major promoter of angiogenesis. Using MMP-2 and MMP-9 knock-out mice, the switching from the quiescent to the angiogenic stage was found to be due to MMP-9 activity. MMP-2 deficiency did not impair the angiogenic switch but reduced the rate of tumor growth.\textsuperscript{31} In the tumor tissue the main source of MMP-9 appeared to be the tumor-infiltrating inflammatory cells.\textsuperscript{32,33} Even though MMP-9 is not solely responsible for the angiogenesis, it’s clear that MMP-9 has an important role in angiogenesis.

B. Detection of Biomarkers in Breast Cancer

Caspase-3 and MMP-9 are important biomarkers in apoptosis and angiogenesis respectively.\textsuperscript{34} The progression of breast cancer can be determined by the accurate detection of these enzymes. Many molecular imaging studies have been reported for the
detection of activity of caspase-3 and MMP-9 enzymes. For the detection of caspase-3 activity by the noninvasive and repetitive imaging of apoptosis in living animals, a reporter was engineered for bioluminescence imaging wherein the estrogen receptor regulatory domain (ER) was fused to FLuc, thereby sterically silencing FLuc catalytic bioluminescence activity. Inclusion of a DEVD sequence between these two moieties allowed for caspase-3-mediated restoration of Fluc activity, enabling real-time monitoring of apoptotic activation. Using this reporter, the investigators demonstrated activation of caspase-3 in intact cells and living animals in response to treatment with TNF-α-related apoptosis-inducing ligand (TRAIL).

Apoptosis has also been detected using near-infrared (NIR) optical imaging of caspase-3 activity and annexin V. A magnetic nanoparticle containing a biotinylated caspase-3-specific peptide substrate (P2), was prepared and incubated with avidin-P1 to form a caspase-3-sensitive magnetic nanoassembly. The peptide substrate was specifically recognized by caspase-3 and thus served as an assay for this enzyme. The caspase-3-mediated reaction was associated with a dose dependent increase in the T2 relaxation time with kinetics similar to those reported with fluorogenic substrates. In yet another study, a FRET-based fluorescence probe for caspase-3 was developed by attaching NIRQ750 and a NIR fluorochrome, Alexa680, to either end of a peptide sequence that
can be cleaved by caspases-3. In the presence of caspase-3, the fluorescence signal of the probe increased about four-fold, and there was no activation in the presence of a caspase-3-specific inhibitor. Caspases are earlier biomarkers of apoptosis, and there are multiple targets in the apoptosis metabolic cascade in addition to caspase-3.

Research has been reported that detects MMP enzymes, especially MMP-2 and MMP-9. These two enzymes cleave the middle of specific peptide sequences and are classified as endo-peptidases. Many verified peptide substrates are cleaved by both enzymes and the specificity of substrates for each enzyme is an issue in the design of molecular probes.

For the detection of MMP-9, the use of near-infrared optical imaging techniques using fluorescence resonance energy transfer (FRET) to visualize matrix-metalloproteinase and cathepsin B can improve the assessment of tumor aggressiveness and angiogenesis-inhibitory therapy. A variation of these probes worthy of note includes quenched near-infrared fluorochromes that can be activated by proteases such as MMP-2. Such a molecular probe consists of three structural elements: A quenched NIR fluorochrome, a poly-L-lysine backbone, and methoxy polyethylene glycol (MPEG) side chains. Coupled to this backbone are specific synthetic peptide substrates containing motifs that can be cleaved by MMP-2. The MMP inhibitor was synthesized and modified for labeling with $[^{123}\text{I}]$ iodine or $[^{125}\text{I}]$ iodine. Overexpressed MMP activity was detected by use of
radiolabeled imaging probes and scintographic imaging modalities (SPECT, PET).

2. MRI and MR Contrast

A. General Concept of MRI

All nuclei consist of protons or neutrons or a combination of both. Nuclei with an odd number of protons, such as $^1\text{H}$, $^{19}\text{F}$, $^{23}\text{Na}$, $^{31}\text{P}$, have a net ionic charge distribution due to the unpaired nucleon, which produces a magnetic moment. Also, a nucleus possesses angular momentum due to its spinning around a central axis. This is commonly referred to as nuclear spin or spin. The spin number is in multiples of half. For example the $^1\text{H}$ atom, with one unpaired proton, and zero electrons has a spin number equal to $\frac{1}{2}$. When placed in a magnetic field of strength $B_0$, a particle with a net spin can absorb a photon of frequency, $\nu$, which is termed as the Larmor frequency, that depends on the gyromagnetic ratio, $\gamma$ (hydrogen, $\gamma = 42.58 \text{ MHz} / \text{T}$).

$$\nu = \gamma B_0$$

(Eq. 1.1)

Considering a macroscopic view of nuclear spins, the spins will distribute among two possible energy states, parallel or anti-parallel to the external magnetic field $B_0$. The parallel state is a lower energy state. The population ratio of protons in the two energy states is determined by the Boltzmann distribution (equation 1.2). The summation of spins in the sample yields a net macroscopic magnetization vector that is aligned with the
external magnetic field. The net magnetization vector can be detected by applying a radio frequency (RF), the Larmor precessional frequency, in a direction that is perpendicular to the net magnetization.

\[ \frac{N_-^o}{N_+^o} = \exp\left(-\frac{\Delta E}{kT}\right) \]  
(Eq. 1.2)

- $N_-^o$: the thermal equilibrium populations of protons in the $m = - \frac{1}{2}$ states
- $N_+^o$: the thermal equilibrium populations of protons in the $m = + \frac{1}{2}$ states
- $\Delta E$: the energy difference between the two states
- $k$: the Boltzmann constant.
- $T$: the absolute temperature in Kelvin of the surrounding lattice.

The RF pulse produces an oscillating magnetic field in a plane perpendicular to the sample magnetization vector. The oscillating field will tip the magnetization away from its thermal equilibrium position due to energy absorptions that transition spin states from lower to higher energy. The magnitude of the transverse field is usually small compared to the static magnetic field $B_0$, however if the RF pulse lasts for a time large compared to the Larmor precessional period, it can significantly nutate the net magnetization such that the entire magnetization can be tipped from alignment with $B_0$. The magnetization, $M_0$, will then precess around $B_0$ at the resonance, Larmor frequency. The macroscopic magnetization that now lies in the transverse plane can be detected by a coil placed
perpendicular to its axis of rotation. Once the net magnetization is tipped away from alignment with the static external magnetic field, it has both a longitudinal ($M_Z$) and transverse ($M_{XY}$) component. The process that returns the longitudinal component to the thermal equilibrium is termed as spin-lattice relaxation ($T_1$ relaxation) and the process that describes the change of transverse magnetization from a non-zero to zero value is termed as spin-spin relaxation ($T_2$ relaxation). Once the RF energy perturbation ceases the transverse magnetization will decay to zero as the spin system returns to its equilibrium state, inducing an electromagnetic frequency (emf) in the detection coil. This process is termed as free induction decay (FID).^51

---

**Figure 1.1** The tipping and relaxation of magnetization $M_0$ relative to $B_0$
B. $T_1, T_2$ relaxation times and contrast

The time constant that describes how $M_Z$ returns to its equilibrium value is called the spin-lattice relaxation time ($T_1$ relaxation). For $T_1$ relaxation, the time required to establish thermal equilibrium between the spins and their surroundings is most efficient, i.e. shortest, when the field fluctuations are on the order of the Larmor frequency. The governing equation of this behavior is given;

$$M = M_0 \sin \theta \frac{1 - e^{-t/T_1}}{1 - \cos \theta e^{-t/T_1}} \quad (Eq. 1.3)$$

for $T_1$ saturation with 90 degree pulse ($\theta = 90^\circ$)

$$M_z = M_0 \left(1 - \exp\left(-t/T_1\right)\right) \quad (Eq. 1.4)$$

The fluctuating fields may be produced by dipole-dipole interactions between nuclear spins as they tumble due to random thermal motion. If the lattice molecules are tumbling very slowly, i.e. correlation times are long, there will be few field fluctuations that are at the Larmor frequency. On the other hand, rapid thermal molecular motion is associated with short correlation times i.e. a wide range of frequencies that consequently have lower spectral power due to conservation of total thermal energy.

The time constant that describes the return to equilibrium of the transverse magnetization, $M_{xy}$, is called the spin-spin relaxation time, $T_2$ relaxation. The spin-spin relaxation
process results in loss of coherence of $M_{xy}$. In some cases, $T_1$ relaxation affects $T_2$ relaxation, i.e. a spin flip with net energy transitions. The loss of transverse coherence is due to a phase dispersion of the nuclear spins as they experience slightly different local magnetic fields. It is in fact the static or zero-frequency component of the power spectrum of local fluctuating fields that is the dominant factor in determining $T_2$ relaxation time. The governing equation is given;

$$M_{xy} = M_0 \sin \theta e^{-t/T_2} \quad \text{(Eq. 1.5)}$$

for $\theta = 90^\circ$,

$$M_{xy} = M_0 e^{(-t/T_2)} \quad \text{(Eq. 1.6)}$$

$T_2$ relaxation is always less than or equal to $T_1$ relaxation. The net magnetization in the XY plane decreases to zero and the longitudinal magnetization increases until $M_z$ equals $M_0$. Both $T_1$ and $T_2$ are specific to the type of tissue and it is these parameters that are used as the basis for image contrast in many MRI exams.

Contrast in MR images is achieved based on differences in $T_1$, $T_2$, and proton density within a sample. Using different RF pulse sequences, image intensity can be weighted with respect to $T_1$, proton density, or $T_2$. Inherent contrast can be improved with the use of paramagnetic contrast agents. Most contrast agents reduce $T_2$ and $T_1$. Agents are
classified as T1 agent if $\Delta T_2 / \Delta T_1 < 4$, and T2 agent if $\Delta T_2 / \Delta T_1 > 4$. The ability to reduce $T_1$ and $T_2$ relaxation times is described by the concentration normalized relaxivity $r_1$ and $r_2$, respectively.

Paramagnetic contrast agents contain a metal ion that has one or more unpaired electrons; the most common agents contain the lanthanide ion gadolinium (III). Paramagnetic relaxation of water protons originates from dipole-dipole interactions between the nuclear spins of the hydrogen nuclei of water molecules and the fluctuating local magnetic field caused by the spins of the unpaired electrons. Observed $T_1$ is due to a combination of the $T_1$ intrinsic to the diamagnetic sample and the $T_1$ caused by the presence of a paramagnetic species (equation 1.7). The term $1/T_{1p}$ can be written as the product of the relaxivity, $r_1$, and the concentration of the contrast agent, [Gd] (equation 1.8). Relaxivity is accepted by interactions between the paramagnetic ion and water in an inner sphere and an outer sphere (equation 1.9). Water molecules in the inner sphere are directly bound to the paramagnetic ion, while water molecules in the outer sphere are closely diffusing water molecules that interact with the paramagnetic ion. The relaxation of current clinically approved agents is due to approximately 60% inner sphere and 40% outer sphere effects. Inner sphere effects can be modified whereas outer sphere effects cannot easily be affected.
The inner sphere effects are dependent on the longitudinal proton relaxation rate, \( \frac{1}{T_{1m}} \), and the rotational correlation time, \( \tau_m \) (equation 1.10). The term \( \frac{1}{T_{1m}} \) represents dipole-dipole interactions and a scalar interaction (equation 1.11). The scalar interaction becomes negligible at magnetic field strengths above 10 MHz. Since most clinical and experimental MR images are acquired at field strengths higher than 10 MHz, the scalar interaction is not an important factor in proton relaxation, thus \( \frac{1}{T_{1m}} \) is essentially determined by dipole-dipole interactions. The dipole-dipole interactions are modulated by reorientation of the nuclear spin vectors with respect to the electron spin vector, changes in orientation of electron spin, and the rate of water exchange (equation 1.12 and 1.13).

\[
\frac{1}{T_{1obs}} = \frac{1}{T_{1d}} + \frac{1}{T_{1p}} \quad \text{(Eq. 1.7)}
\]

\[
\frac{1}{T_{1obs}} = \frac{1}{T_{1d}} + r_i [Gd] \quad \text{(Eq. 1.8)}
\]

\[
r_i = r_i^{IS} + r_i^{OS} \quad \text{(Eq. 1.9)}
\]

\[
\frac{1}{T_i^{IS}} = \frac{eq}{55.5} \left( \frac{1}{T_{1n} + \tau_n} \right) \quad \text{(Eq. 1.10)}
\]

\[
\frac{1}{T_i^{DD}} = \frac{1}{T_i^{DDo}} + \frac{1}{T_i^{DDc}} \quad \text{(Eq. 1.11)}
\]

\[
\frac{1}{T_i^{DDo}} = \frac{2}{15} \left( \gamma^e \gamma_p \mu_s \right) S(S+1) \left( \frac{\tau_{c2}}{1 + \sigma_s^2 \tau_{c2}^2} + 3 \frac{\tau_{c3}}{1 + \sigma_s^2 \tau_{c3}^2} \right) \quad \text{(Eq. 1.12)}
\]
The theory of T1 contrast agents demonstrates that numerous parameters affect relaxivity (Figure 1.2). The parameters \( q, r_{\text{GdH}}, \tau_m, \tau_r, \) and \( T_{1e} \) can be adjusted by altering the chemical environment around the paramagnetic ion.\(^{52}\) By increasing the value of \( q \), the relaxivity of the agent will increase. Increasing \( q \) above two will most likely result in increased toxicity due to decreased stability of the complex. A decrease in \( r_{\text{GdH}} \) will lead to an increase in relaxivity. Decreasing the term \( \tau_m \) will allow more water molecules to be affected by the gadolinium (III) ion within a time frame dictated by \( T_1 \) resulting in an

\[
\frac{1}{T_{1i}} = \frac{1}{\tau_c} + \frac{1}{\tau_r} + \frac{1}{T_{1e}}, i = 1, 2
\]  

(Eq. 1.13)

\[c\] the molal concentration of contrast agent
\[q\] the number of bound water molecules per paramagnetic ion
\[\tau_m\] the mean lifetime of water molecules in the inner sphere environment
\[1/T_{1m}\] the longitudinal proton relaxation rate.
\[\gamma_l\] the nuclear gyromagnetic ratio
\[g\] the electron g-factor
\[\mu_B\] the Bohr magneton
\[\gamma_{\text{Gd}}\] the electron spin - proton distance
\[S\] the spin of the paramagnetic ion
\[\omega_S\] the electron Larmor frequency
\[\omega_l\] the nuclear Larmor frequency
\[\tau_c\] the correlation time
\[\tau_m\] the rotational correlation time
\[\tau_r\] the reorientational correlation time of the metal-proton vector
\[T_{1e}\] the electron spin relaxation times of the metal ion

longitudinal (i=1) and transverse (i=2)
increase in relaxivity. If the value of $\tau_m$ is decreased too much, the relaxivity of a complex will begin to decrease because the lifetime of the water molecules bound to the gadolinium (III) ion will not be long enough to influence the relaxation of the protons of the water molecule. By optimizing the value of $\tau_r$ or $T_{1e}$, the relaxivity of the contrast agent will be increased. There is an interdependence of the terms $\tau_m$, $\tau_r$, and $T_{1e}$. For most small molecule gadolinium (III) complexes, $\tau_r$ is the most limiting of the three variables. As the value of $\tau_r$ becomes optimized the variables $\tau_r$ and $T_{1e}$ begin to influence the relaxivity of the contrast agents. These parameters can be exploited to create responsive contrast agents.

**Figure 1.2** Schematic of the interactions of water molecules with a lanthanide (III)-based contrast agent. The water accessibility ($\tau_m$), rotational tumbling time ($\tau_r$), electron spin state ($T_{1e}$), chemical exchange rate ($\tau_{ex}$), MR frequency ($\delta$) are shown, but RF inhomogeneity is not shown in this scheme.
C. Paramagnetic Chemical Saturation Transfer (PARACEST)

Chemical Exchange Saturation Transfer (CEST) involves chemical exchange of a nucleus in the NMR experiment from one site to a chemically different site. As the name suggests, CEST considers the transfer of saturation in a system that is undergoing chemical exchange. In order for an effect to be observed, the exchange process must occur between two magnetically distinct environments and must be slow on the NMR timescale. "Slow" means that the rate of exchange ($k_{ex}$) must be no greater than the difference in frequency between the two chemical environments ($\Delta \omega \gg k_{ex}$).

In a system that meets these conditions for two pools of exchanging protons, Pool A and Pool B, saturation of the protons in Pool B also has an effect on the protons in Pool A. The saturation pulse increases the number of high energy spins aligned against the field in Pool B, thereby decreasing the bulk magnetization in this Pool. The spins in Pool A initially retain their normal Boltzmann equilibrium but, with time, chemical exchange perturbs this situation. The exchange process can be considered as two independent equilibria with identical forward and reverse rate constants (Figure 1.4). The first equilibrium is that between the low energy states (a) of Pool A and Pool B and this will move to increase the number of spins in the low energy state of Pool B at the expense of those in Pool A. The second equilibrium is that between the two high energy states (b)
and this will move such that the number of high energy spins in Pool A is increased at the expense of the number of high energy spins on Pool B. Thus, the result of chemical exchange is that the number of high energy spins in Pool A increases while the number of low energy spins in this pool decreases. This CEST process is in competition with relaxation. If the longitudinal relaxation rate ($R_1$) of Pool B is faster than the forward rate constant of the exchange process (Pool B $\rightarrow$ Pool A) then the system will relax back to the Boltzmann distribution before exchange can transfer the altered spin state to Pool A. In this situation a normal NMR spectrum will be obtained. Similarly, if the relaxation rate of Pool A is rapid, the system will have relaxed back to its Boltzmann distribution prior to interrogation of the system. So in addition to suitable exchange kinetics, a CEST effect will only be observed if the relaxation times of the two pools are long relative to the exchange rates. So the signal intensity of one resonance can be altered by applying a presaturation pulse to a second resonance in exchange with the first.

The paramagnetic complexes used as traditional $T_1$-shortening agents have at least one water molecule coordinated to the metal center that exchanges relatively rapidly with bulk water. One class of paramagnetic lanthanide complexes that had been ruled out as $T_1$-shortening agents because water exchange was too slow were complexes formed with tetraamide derivatives of DOTA. Water exchange in some lanthanide DOTA-tetraamide
(DOTAM) complexes has been observed to be so slow that a separate resonance for the lanthanide-coordinated water protons can be observed with larger chemical shift away from the solvent water peak. If $\Delta \omega$ is approximately 10-fold larger in the system, the maximum permitted exchange rate is also enhanced by a factor of 10.
The protons of the coordinated water molecule are not the only exchangeable protons. The MR frequency of amide protons are not shifted so strongly as those of the coordinated water molecule in the case of the europium-DOTAMGly complex, but they are easily detected in the spectra of other lanthanide complexes with DOTAMGly, such as those formed with ytterbium or dysprosium. The CEST spectrum of lanthanide (Yb, Tm, Er, Ho, Dy, Eu)-DOTAMGly shows a strong CEST effect arising from amide proton exchange at -16, -51, -22, 39, 77 and -4 ppm. There are two principal reasons why the
amide protons give rise to a larger CEST effect. First, the coordinated water protons of Ln-DOTAMGly are more effectively relaxed by the dysprosium ion than the amide protons. Secondly, there are eight exchanging amide protons in this complex compared to only two water protons. Hence, more exchanging protons translate to a larger CEST effect.

The hyperfine shift characteristics and relaxation properties of a PARACEST agent can be tuned according to the choice of lanthanide ion. Indeed the resonance chosen for CEST activation can be shifted either upfield or downfield of solvent water with varying magnitude depending upon the lanthanide ion chosen. This means that more than one PARACEST agent could be administered simultaneously and each could be activated separately by an appropriate choice of CEST frequency.
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Chapter II. Responsive MR contrast agents
1. **Introduction**

During the last 30 years, Magnetic Resonance Imaging (MRI) has developed from an intriguing research project to an essential diagnostic method in the armamentarium of clinical radiologists. An estimated 26.6 million MRI examinations were performed annually in 2006, with a 3% annual increase in the number of MRI exams since 2003. The growth of MRI is partly driven by the broad variety of clinical examinations that exploit a variety of MRI contrast mechanisms in endogenous tissues. Different image contrast generated by different soft tissues can be used to assess anatomy at excellent spatial resolution that is typically less than one 1 mm. Different image contrast can also assess physiological function, such as the function of the cardiopulmonary system (e.g., MR angiography of vasculature), neurological system (e.g., fMRI of brain activity), renal system (e.g., perfusion imaging of kidney function), musculoskeletal system (e.g., MR elastography of connective tissues), and cancer lesions (e.g., Dynamic Contrast-Enhanced MRI of angiogenic tumors).

Although MRI contrast in endogenous tissues provides excellent sensitivity for detecting subtle changes in anatomy and function, MRI has poor specificity for attributing image contrast to pathologies. For example, MRI matches or surpasses X-ray mammography in its ability to identify breast cancer lesions, but MRI also detects many non-cancerous
lesions that lead to erroneous false-positive diagnoses, so that MRI breast exams are currently only recommended for women with high cancer risk factors. In addition, the changes in anatomy or function that are detected with MRI are often the consequence of mid- to late-state development of the pathology, which are too late for the application of preventative or early-stage treatments. To meet these needs, MRI methods are being developed that cause changes in MR image contrast in response to molecular compositions and functions that serve as early biomarkers of pathologies. These new MRI methods comprise a major role in the paradigm of molecular imaging.

2. **Exogenous MR contrast agents**

Most molecular imaging studies critically depend on the development of exogenous agents that change image contrast in response to molecular compositions or functions within *in vivo* tissues. Exogenous T$_1$ relaxation agents typically contain Gadolinium, which has a large spin-7/2 magnetic dipole moment and seven unpaired electrons, and therefore has excellent characteristics to accelerate T$_1$ relaxation of water molecules that are near the Gadolinium metal ion. Exogenous T$_2^*$ relaxation agents typically contain iron oxide nanoparticles that are superparamagnetic and cause local magnetic field inhomogeneities that accelerate T$_2^*$ relaxation of water molecules that are near the iron
oxide nanoparticle. Exogenous CEST agents typically contain paramagnetic lanthanide ions other than Gadolinium, which shift the MR frequencies of a CEST agent’s hydrogen nuclei. Large frequency shifts of these PARAMagnetic CEST (PARACEST) agents greatly facilitate selective excitation and also allow for faster hydrogen exchange rates with water that improves detection sensitivity.

Many responsive MRI contrast agents are designed to bind to endogenous molecules in a reversible manner that only involves non-covalent interactions, such as peptides that bind to cell receptors or extracellular matrix components. Reversible responsive MRI contrast agents can change their $T_1$ relaxation upon binding to their target molecule, by reducing their rotational tumbling rate or water accessibility, or by changing the electron spin state of the agent. Similarly, the binding of many small iron oxide nanoparticles to a molecular target can create a larger superparamagnetic nanoparticle, which increases local magnetic field inhomogenities and decreases $T_2^*$ relaxation times. A PARACEST agent can show an altered chemical shift or hydrogen exchange rate after binding to a target molecule.

MRI contrast agents are relatively insensitive, requiring a minimum threshold of 10-10,000 μM for adequate detection during in vivo applications. Endogenous metabolites that are present at concentrations that are higher than the detection threshold are feasible targets for this approach. Proteins and nucleic acids are typically present at much lower
concentrations in the range of nM, and therefore these reversible responsive agents have been difficult to use to target proteins and nucleic acids. For comparison, in vivo PET, SPECT, and fluorescence imaging typically have detection sensitivity thresholds of pico molar concentrations (10^{-3} nM), and are more practical for detecting proteins and nucleic acids through a reversible binding mechanism. Newer approaches that load MRI contrast agents onto nanoparticles may overcome this sensitivity problem, but at the expense of altered pharmacokinetics and potential toxicities. In addition, the specificity of the non-covalent binding for the desired target molecule relative to other potential targets must be carefully evaluated, in order to confidently interpret the response of the reversible MRI contrast agent.

Alternatively, responsive MRI contrast agents may be designed to undergo irreversible covalent changes during interactions with endogenous molecules, such as agents that are substrates for enzymes. T_1 relaxation of an irreversible responsive MRI contrast agent can be changed through enzymatic cleavage of a ligand that blocks water accessibility to the agent’s metal ions. These MRI contrast agents can change their T_1 relaxation or T_2* relaxation by undergoing enzymatic polymerization or depolymerization that changes the agent’s rotational tumbling rate. Polymerization or depolymerization can also alter the association of small iron oxide nanoparticles that form a larger superparamagnetic
nanoparticle. PARACEST agents can undergo covalent changes that change chemical functional groups, which exhibit altered hydrogen exchange rates and MR frequency of the exchangeable hydrogens in the functional group. Irreversible responsive contrast agents can exploit a high catalytic turnover rate from a relatively low concentration of target enzyme to generate a high concentration of responsive agent that is above the MRI detection threshold. The high specificity of the enzyme reaction may also lend confidence when interpreting the response of an irreversible MRI contrast agent.

In order to translate changes in image contrast to these changes in $T_1$ relaxation, $T_2^*$ relaxation, or the PARACEST effect, other characteristics that change image contrast must be constant or monitored by other methods. In particular, the concentration of the contrast agent affects image contrast, and \textit{in vivo} pharmacokinetics rarely allow for constant tissue concentrations of contrast agents during the MRI scan session, so that the concentration of the contrast agent must be monitored. This may be accomplished by adding a second, unresponsive contrast agent that has identical pharmacokinetics or that is covalently linked to the responsive contrast agent. This poses a daunting problem for MRI contrast agents that depend on $T_1$ and $T_2^*$ relaxation, because the $T_1$ or $T_2^*$ contrast can only monitor one agent during the MRI scan session, and the two types of relaxation too correlated to distinguish one effect from the other during the same MRI scan session.
The addition of a second, unresponsive contrast agent during the same scan session is feasible with PARACEST MRI, because PARACEST agents can be selectively detected via different saturation frequencies.

3. **Responsive MR contrast agents**

   **A. Responsive MRI contrast agents for molecular imaging of proteins**

Proteins are responsible for a broad variety of molecular compositions and functions, and therefore serve as excellent biomarkers for many pathologies. The proteome consists of 303,907 proteins,\(^1\) and there are 21,688 proteome and 3051 proteins estimated druggable targets for human and approximately 600~1500 are considered to be molecular targets of potential drug therapies for humans. Extracellular proteins are more accessible to exogenous contrast agents than intracellular proteins. Although transmembrane delivery of exogenous contrast agents to target intracellular proteins is a pharmacokinetic barrier, the barrier of transmembrane ‘escape’ of a responsive agent can aid in localizing and retaining the agent at the site of the protein target.

   **a. Contrast agents that bind to proteins**

Due to the relative insensitivity of MRI, responsive contrast agents are generally limited to targeting proteins that are present at high concentrations, such as proteins that
contribute to connective tissues and proteins that residue in the blood pool at high concentrations. For example, a phosphonated Gd chelate, GdDOTP\textsuperscript{5}, is designed to bind to hydroxyapatite, which reduces water accessibility and decreases the $T_1$ relaxation of the agent.\textsuperscript{2} Hydroxyapatite is a major component of healthy bone tissue, so that this agent can be used to bone lesions that are devoid of hydroxyapatite. As another example, the rotational tumbling rate of a biotinylated contrast agent, DO3A-EA, is reduced when bound to the larger avidin protein that is administered to the patient, so that the MRI contrast responds to high concentrations of avidin in the cardiovascular system.\textsuperscript{3}

Similarly, another contrast agent, MS-325, binds to the albumin that resides in the blood pool at high concentration, which slows the tumbling rate of the contrast agent upon binding to this larger protein.\textsuperscript{4} A contrast agent with a peptidyl ligand has been designed to bind to the Gal80 glucose storage protein, which also slows the tumbling rate of the agent.\textsuperscript{5} Carbonic anhydrase has been selectively targeted by Gd-DTPA-sulfonamide ethylene sulfanilamide, which has a slow tumbling rate after binding to its protein target.\textsuperscript{6} The reduced tumbling rates lead to improved $T_1$ relaxations for each agent when bound to the target protein. For each of these cases, care must be taken to ensure that each binding agent is specific to the intended protein target.

Other examples have shown that antibodies and peptides that are labeled with MRI
contrast agents can bind overexpressed protein cell receptors that are present at high concentrations. Although some examples are promising candidates for detecting these cell receptors, the MRI contrast is dependent on the accumulation of the agent, and not a response caused by a change in physical properties of the agent.

**b. Contrast agents that are catalyzed by enzymes**

Targeting enzymes provides several important advantages for the design of responsive MRI contrast agents. First, the high catalytic rate of a relatively low concentration of enzyme can develop a relatively high concentration of altered contrast agent, so that MRI sensitivity is less problematic for enzyme detection. Second, the specificity of enzymatic reactions is usually high, so that a change in MRI contrast can often be confidently attributed to the specific targeted enzyme. Lastly, enzymatic activity can cause a variety of irreversible responses in a contrast agent, which can be exploited to develop many types of responsive MRI contrast agents.

One of the seminal examples of a responsive MRI contrast agent exploits a change in water accessibility after β-Galactosidase enzymatically cleaves a galactopyranose ligand from the contrast agent. More recently, the hydrogen exchange rate and MR frequency of a PARACEST agent has been shown to be altered after a peptidyl ligand of the agent is
cleaved by caspase-3. The rotational tumbling rate can be changed after a phosphate monoester ligand of a contrast agent is hydrolyzed to an alcohol by alkaline phosphatase, which can then more easily bind to albumin. Only 4.3 μM, 3.4 nM, and 3 units of each respective enzyme was required to generate sufficient MRI contrast in a practical time frame, and high specificity for each respective contrast agent substrate was shown to be excellent.

The rotational tumbling rate of the contrast agent can be changed by polymerizing monomeric agents. Polymerization of phenolic contrast agents has been exploited to detect several peroxidases, including myeloperoxidase an oxidoreductase. The decrease in tumbling rate caused by the polymerization is sufficient to cause a three-fold increase in T₁ relaxivity. The polymerization also aids in retaining the contrast agent at the target site, either through slower pharmacokinetics of the polymeric form of the agent, or by cross-linking the agent to the local extracellular matrix. As another example, a stearic acid ligand of a contrast agent can be cleaved by an esterase.

Degradation of a polymer can also change the rotational tumbling rate of a contrast agent. This mechanism is typically exploited by conjugating contrast agents with a linker that is cleaved by a specific enzyme. For example, a glucuronide linker is cleaved by glucuronidase, and a hyaluronan linker is cleaved by hyaluronidase, which
releases Gadolinium chelate from the polymer. The relatively rapid pharmacokinetics of monomeric contrast agents can accelerate removal of the agent from the site of degradation, which can further enhance the response of the agent to degradative enzyme activity.

Polymerization or degradation can also be exploited to change the association of iron oxide nanoparticles that cause local field inhomogenities, which has been termed as a ‘magnetic relaxation switch’. Peroxidase-induced polymerization of phenolic contrast agents can assemble dextran-coated iron oxide nanoparticles to create an even stronger superparamagnetic particle that decreases the $T_2^*$ relaxation time. Caspase-3-induced degradation or MMP-2-induced degradation of a short peptide sequence that links multiple avidin-biotin-$T_2^*$ contrast agents can increase the $T_2^*$ relaxation time. Degradation of a double-stranded DNA linker by DNA methylation & cleavage enzymes can also cause an increase in $T_2^*$ relaxation time.

**B. Responsive MRI contrast agents for molecular imaging of nucleic acids**

The genomics revolution has identified an abundance of nucleic acid targets among 24,000 human genes that are potential biomarkers of pathologies. The strong interactions between complimentary nucleic acid sequences can provide very high selectivity for
detecting a specific nucleic acid target. The ‘magnetic relaxation switch (MRS)’ method has been successfully applied to detect the DNA sequence for Green Fluorescence Protein in biochemical solutions within well plates, versus DNA sequences with single mismatches relative to the target DNA sequence.\textsuperscript{22} This method exploits the aggregation of iron oxide-labeled nucleic acid sequences that bind to a portion of the target DNA sequence, which increases local field inhomogeneities and decreases $T_2^*$ relaxation time. Yet directly detecting a specific nucleic acid sequence within the in vitro or in vivo context is a daunting challenge for MRI, primarily due to the extremely low concentration of specific DNA and RNA sequences that are present at <10 and <100 copies within each cell, respectively. The intranuclear and intracellular locations of nucleic acids pose the additional problem of intracellular delivery and trafficking of the DNA-targeting contrast agent.

A more promising approach is the use of a reporter gene imaging strategy to study gene expression during \textit{in vitro} or \textit{in vivo} studies. This strategy requires transgenic manipulation of the cell or animal model to include two genes that are always co-expressed. One of the genes is the ‘target gene’ that is the focus of the gene expression study. The other gene represents the ‘reporter gene’ that encodes for a ‘reporter protein’ that can be detected by a responsive MRI contrast agent using one of the many
mechanisms discussed in section 2. A responsive MRI contrast change indicates expression of the ‘target gene’. Reporter gene imaging with optical and nuclear imaging modalities has become tremendously successful, and similar successes with MRI reporter gene strategies are likely to emerge in the near future.

C. Responsive MRI contrast agents for molecular imaging of metabolites

Many metabolites exist at high concentrations within an in vitro or in vivo environment, which greatly facilitates their detection by responsive MRI contrast agents. The variety of molecular structures provides opportunities to exploit several mechanisms that are employed by responsive MRI contrast agents. Yet this variety of molecular structures is a challenge when designing responsive MRI contrast agents, so that first-generation agents may only weakly interact with the target metabolite. In addition, care must be taken to selectively detect one of many similar metabolites to ensure the selectivity of the MRI response for the target metabolite.

PARACEST agents are insensitive and require high concentrations of their target, but can be very responsive to weak interactions. Therefore, PARACEST agents are well suited for the detection of metabolites. The hydrogen exchange rate of a PARACEST contrast agent has been shown to change when the agent noncovalently binds to a sugar, although
selectivity for specific sugars is marginal. The MR frequency of a PARACEST contrast agent changes from -29.1 ppm to -15.5 ppm when the agent noncovalently binds to L-lactate. Both the hydrogen exchange rate and the MR frequency of a PARACEST contrast agent change when the agent undergoes an irreversible covalent reaction with nitric oxide (Liu, Li, and Pagel, unpublished results). In each case, care must be taken to ensure that the interactions between high concentrations of the PARACEST agent and the metabolite do not perturb the biological system.

Fewer mechanisms have been exploited to change \( T_1 \) or \( T_2^* \) relaxation in response to metabolites. Water accessibility can be altered when glucose binds to a Gadolinium chelate. Degradation of thiol-containing polymeric contrast agents can be caused by dithiothreitol or primary radicals \( \text{OH}^\cdot, \text{H}^\cdot, \text{HO}_2^\cdot, \text{H}_3\text{O}^+, \text{O}_2^-, \) or \( \text{e}^-_{\text{aq}} \). Further research to improve the strength of interactions between contrast agents and specific metabolites is required to exploit additional responses in \( T_1 \) or \( T_2^* \) relaxation.

**D. Responsive MRI contrast agents for molecular imaging of oxygen**

The partial pressure of oxygen, \( p_{\text{O}_2} \), is relevant to many pathologies including cerebral infarcts and tumor tissues. The oxidation state of many metal ions is dependent on \( p_{\text{O}_2} \), which can be exploited to change the response of MRI contrast agents relative to \( p_{\text{O}_2} \).
Manganese complexes of 5,10,15,20-tetrakis-(p-sulfonatophenyl) porphinate change their redox state between Mn$^{\text{II}}$ and Mn$^{\text{III}}$ depending on pO$_2$. The relaxivity of Mn$^{\text{III}}$ complex is mainly affected by the electronic relaxation time and that of Mn$^{\text{II}}$ complex is mainly controlled by the rotational motion of the complex. Poly β-CD is used to avoid the aggregation of phophyrine complexes and enhance the stability Mn$^{\text{II}}$ complexes.$^{27}$

The iron in the porphorin ring of hemoglobin changes redox state from diamagnetic Fe$^{\text{II}}$ to paramagnetic Fe$^{\text{III}}$ when bound to oxygen. The injection of 2~5 μL of 8 mM of hemoglobin can show pO$_2$-dependent changes in T$_2$-weighted MR images as large as 50%.$^{28}$

In addition to a change in electron spin state, the rotational tumbling rate can also be changed in response to pO$_2$. The boronic functionalities of Bis($m$-boroxyphehylamide)-(Gd-DTPA) bind to fructosamine on the glycated surface of oxygenated hemoglobin, but have less affinity for binding deoxygenated hemoglobin.$^{29}$ The change in rotational tumbling rate upon binding causes a decrease in T$_1$ relaxation time.

**E. Responsive MRI contrast agents for molecular imaging of metal ions**

Metal ions, especially divalent metal ions, are associated with many biological signaling pathways and pathologies. The rapid flux of metal ions on a sub-second time scale can
require rapid measurements, which can pose a particular challenge for accurate quantification through MRI contrast changes. As with pO₂ measurements, the measurements of metal ion concentrations must be conducted in physiologically relevant concentration ranges. Consideration should also be given for ensuring that the MRI contrast agent detects the desired metal, although metal binding motifs often show excellent specificity for one type of metal.

Chelates that bind metals are often incorporated into contrast agents to change MRI contrast in response to metal concentrations. For example, calcium can be chelated by four carboxylates to form a complex with a dissociation constant of approximately 0.96 μM. A calcium-responsive MRI contrast agent has been designed with two Gd-DOTA moieties linked by a bis-anilide bridge that contains four carboxylates. When calcium is absent, the carboxylates bind to the Gadolinium ions, which inhibit water accessibility and increases T₁ relaxation time. Iron-responsive MRI contrast agents with a phenanthroline-like chelator moiety have been used to create tris complexes or trinuclear structures that have increased rotational tumbling times and decreased T₂* relaxation times. Another iron-responsive MRI contrast agent has a 2,2’-bipyridine moiety that binds to Fe²⁺ ions and then self-assembles into a metallostar [Fe{Gd₂L(H₂O)₄}₃]⁴⁻ structure that has a slower rotational tumbling time. A zinc-
responsive MRI contrast agent with pyridine donors shows a faster hydrogen exchange rate and improved PARACEST when the pyridines chelate zinc. The chelation causes a subtle conformational change from a square-antiprism (SAP) geometry to a twisted-square-antiprism (TSAP) geometry, which facilitates the exchange of bulk water with the water molecule directly bound to the lanthanide of the agent.35

Proteins and peptides can reversibly associate with metals to trigger intermolecular associations. This biochemical mechanism has been exploited to generate aggregates of the iron-labeled calmodulin protein and iron-labeled M13 peptide in the presence of calcium, with a subsequent decrease in $T_2^*$ relaxation time.36 This example shows the potential creativity in exploiting mechanisms in biochemistry and molecular biology as additional steps to change MRI contrast in response to molecular biomarkers.

F. Responsive MRI contrast agents for molecular imaging of pH

Assessments of altered pH can be used to diagnose the progression of many pathologies, including renal failure, ischemia, and chronic obstructive pulmonary disease, and can also be critical for developing therapies that are effective in tissue environments with altered pH. Measuring altered pH is particularly relevant for cancer assessments, because poor perfusion, increased lactic acid secretion, and reduced bicarbonate levels within tumor
tissues can create high H\(^+\) concentrations within the interstitial fluid. MRI is particularly well suited for evaluating pH variations over small tissue volumes due to its high spatial resolution.

The ideal pH-responsive agent should accurately measure the entire physiological pH range from 6.0 to 8.0 through a monotonic change in T\(_1\) relaxation, T\(_2^*\) relaxation, or PARACEST. The concentration of these pH-responsive agents must be taken into account to assign image contrast to pH values. A pH-unresponsive contrast agent can be used to account for concentration of the pH-responsive agent. Two T\(_1\) or T\(_2^*\) contrast agents must be serially administered, but two PARACEST contrast agents can be selectively detected so that simultaneous administration of two PARACEST contrast agents may be feasible.

MRI contrast agents can include pH-dependent ligands that alter water accessibilities. Gd\(^{3+}\)-DOTA-tetraamide phosphonate (Gd(DOTA)-4AmP\(^5\)) shows a two-fold increase in T\(_1\) relaxation from pH 8.5 to 6.0. The hydrogen-bonding network created by the protonated phosphonates is believed to provide a catalytic pathway for exchange of the bound water protons with protons of bulk water.\(^{37,38}\) Gd(DOTP)\(^5\) shows no pH dependence, and has be used to account for concentration of the pH-responsive agent to produce high resolution pH maps of rat glioma.\(^{39}\) Water accessibility can also be changed through acid-catalyzed dissociation of a nitrophenol from gadolinium in Gd(NP-
DO3A), while the ‘control agent’ Gd(NP-DO3AM) shows no dissociation and only a modest pH-dependent change in relaxation time.\textsuperscript{40} A pH-dependent sulfonamide ligation of gadolinium can switch the hydration state of this lanthanide, although the binding of endogenous anions or proteins may also affect the hydration state and suppress the expected pH-dependent change in $T_1$ relaxation time.\textsuperscript{41,42} Triaquahexaazamacrocyclic complexes experience a reduction of water accessibility due to coordination of OH\textsuperscript{-} anions, which can be exploited to measure basic pH ranges.\textsuperscript{43} Liposomes consisting of dipalmitoyl phosphatidyl ethanolamine and palmitic acid show pH-dependent stabilities, which can be exploited to cause a change the water accessibility of $T_1$ contrast agents that are encapsulated in these liposomes.\textsuperscript{44,45} A polyion complex swells at low pH, which changes the accessibility of water for the contrast agents within the complex.\textsuperscript{46} MRI contrast agents can experience a change in rotational tumbling time in response to pH changes. Aggregation of Gd@C60(OH)x or Gd@C60[C(COOH)\textsubscript{2}]\textsubscript{10} gadofullerene derivatives occurs at low pH, which causes a decrease in $T_1$ relaxation time as the pH is changed from 12 to 3.\textsuperscript{47} High pH causes deprotonation of phospholipid mimetic structures HADO-(Gd-DO3A) and C18\textsubscript{2}–(Gd-DTPA-Glu), which causes higher lipophilicity that drives formation of colloidal aggregates, causing the $T_1$ relaxation time to decrease at higher pH.\textsuperscript{48,49} A PAMAM dendritic contrast agent exhibits an increase in
rigidity as pH decreases from 11 to 6, causing $T_1$ relaxation time to decrease by 60%.\textsuperscript{50} Self assembled magnetic micelles change hydrodynamic diameter from 200 nm at low pH to 300 nm at high pH, which changes the $T_2$ relaxivity of iron oxide nanoparticles that are encapsulated within the micelle.\textsuperscript{51} A polyornithine polymer exhibits a flexible random coil conformation at low pH and forms a rigid helical conformation at high pH, which decreases the $T_1$ relaxation time of gadolinium chelates that are attached to the polymer.\textsuperscript{52,53} The base-catalyzed exchange of amide hydrogens and water hydrogens is dependent on pH. MRI contrast agents that contain primary or secondary amide groups have shown large increases in PARACEST with increasing pH.\textsuperscript{54-57} This approach has been extended to non-paramagnetic polylysine and PAMAM dendrimers that show a pH-dependent CEST effect.\textsuperscript{58} A pH-unresponsive PARACEST effect may be generated by a metal-bound water molecule, which provides the opportunity to monitor a pH-responsive and pH-unresponsive PARACEST effect from the same contrast agent. The ratio of these two PARACEST effects can provide a concentration-independent measure of pH. A similar ratiometric approach has been applied to the PARACEST effects from the acid-catalyzed hydrogen exchange of an amine group and a base-catalyzed hydrogen exchange of an amide group, which provides a greater dynamic range of PARACEST imaging for
measuring pH (Liu, Li and Pagel, unpublished results).

**G. Responsive MRI contrast agents for molecular imaging of temperature**

MR thermometry techniques have been developed to address pathologies such as heart arrhythmias, and the increasing needs for assessing thermal-based drug delivery and thermal ablation therapies. The high spatial resolution and temporal resolution of MRI makes this modality particularly useful for thermal mapping. Ideally, MRI contrast agents that are responsive to changes in temperature must be very accurate and must not be adversely affected by other environmental conditions such as pH or molecular compositions. The temperature dependence of MR frequencies can meet these conditions. For example, PARACEST thermometry has been performed by identifying the MR frequency that provides the greatest PARACEST effect, and these results have been translated to generate a temperature map.\(^{59}\)

The transmembrane permeability of liposomes can be strongly dependent on temperature. Liposomes that encapsulate Gd(DTPA)-BMA show slow water exchange between the liposome interior and exterior, but this water exchange rate shows a sharp increase as the temperature exceeds the gel-to-liquid crystalline phase-transition temperature of the liposomes.\(^{60}\) Similarly, the exchange of gadolinium- or manganese-containing MRI
contrast agents can more easily escape the interior of the liposomes above the phase-transition temperature. In both cases, the increased water accessibility to the agent leads to a decreased $T_1$ relaxation time.

4. Future directions

These examples of MRI contrast agents demonstrate that MRI contrast mechanisms can respond to molecular compositions and functions through relatively simple changes in water accessibilities, rotational tumbling rates, local magnetic field inhomogeneities, MR frequencies, or hydrogen exchange rates. However, over 10 years of research and development have been invested to produce only 53 examples, and more effort will be required to translate responsive relaxivity-based and PARACEST MRI contrast agents to the clinic.

The dawning of the field of responsive MRI contrast agents has necessarily required extensive design and characterization of unique MRI contrast agents in order to creatively explore the relationships between molecular responses and changes in a MRI contrast agent. This approach has often required the development of specialized synthetic schemes to produce each unique MRI contrast agent. Yet to accelerate the application of responsive MRI contrast agents, a greater focus should be placed on agents that can be
rapidly synthesized with high yield and purity, and that can be applied to large sets of molecular biomarkers. For example, $T_2^*$ agents that are depolymerized by an enzyme can be rapidly synthesized by linking iron oxide nanoparticles with peptide linkers. Similarly, the synthesis of a PARACEST agent with a peptidyl ligand can be accomplished with a commercially available solid phase peptide synthesizer. In both cases, the sequence of the peptide ligand can be chosen to be sensitive to one of many protease enzymes, so that each of these types of responsive MRI contrast agents can be easily applied to detect specific members of the human ‘degradome’.

Because pathologies often exhibit modest changes in the expression, production, delivery, or activity of various biomarkers, an emphasis should be placed on the ability to quantitatively translate the response in MR image contrast to the concentration of the biomarker. Absolute quantifications of MR image contrast is a daunting challenge, because many characteristics of the sample or patient, the instrumentation, and the choice of acquisition parameters and image processing methods can alter image contrast. Ratiometric quantifications are more feasible, in which the response caused by a molecular biomarker is the only effect that is allowed to vary between two MR images, or between two or more regions of the same image. PARACEST may greatly facilitate ratiometric quantifications, because two or more PARACEST agents can be selectively
detected within the same sample volume by saturating their respective CEST MR frequencies. The selective detection of each PARACEST agent also provides opportunities to detect more than one molecular target during a single MRI scan session, which may lead to the diagnoses of “molecular signatures”, or multiple molecular biomarkers of pathological tissues.

In most examples, the direct interaction of a responsive MRI contrast agent and a molecular biomarker has directly led to a change in MRI contrast. Some recent examples demonstrate that more complex mechanisms in molecular biology can be cleverly incorporated to form multi-step processes that indirectly link the interaction of responsive MRI contrast agents and biomarkers with an eventual change in MRI contrast. Reporter gene imaging is an obvious example, in which the steps of gene transcription and translation to form a reporter protein, delivery of an agent to the target tissue (and possibly to the target cell, target intracellular environment and intracellular organelle), and interaction of the agent and reporter protein must each occur before MRI contrast is altered. Additional multi-step mechanisms such as post-translational protein processing, protein-protein interactions and cell-cell signaling are likely to be employed with future responsive MRI contrast agents. Therefore, interdisciplinary research that combines molecular biology with radiology and chemistry will be critical for further developing
responsive MRI contrast agents.
5. References

1. PRIDE PROteomics IDEntifications database: http://www.ebi.ac.uk/pride/


Table 2.1 Structures of responsive MR contrast agents

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Chapter III. Solution Phase Synthesis of Molecular Imaging Contrast Agents

Byunghee Yoo and Mark D. Pagel
A Facile synthesis of α-amino-DOTA as a versatile molecular imaging probe.  
1. Introduction

Macrocyclic metal chelates using DOTA are often administered to in vivo patients and animal models to create or enhance contrast in biomedical molecular imaging studies. Examples include paramagnetic Gd$^{3+}$-DOTA for magnetic resonance imaging (MRI),\textsuperscript{1} radioactive $^{111}$In-DOTA for SPECT imaging,\textsuperscript{2-4} and radioactive $^{64}$Cu-DOTA for PET imaging.\textsuperscript{5-7} More recently, metal-DOTA chelates have been conjugated to peptides to affect the pharmacokinetics of the metal-DOTA imaging agent within in vivo systems, which can be used to gain additional information about biological processes at the molecular level. A variety of peptidyl ligands\textsuperscript{8-10} have been employed for these molecular imaging studies, including peptides that bind to specific cell surface receptors,\textsuperscript{11-13} peptides that penetrate cell membranes,\textsuperscript{14-18} peptides that nonspecifically interact with the extracellular matrix,\textsuperscript{19} and very large peptide homopolymers that drastically alter renal clearance rates.\textsuperscript{20}

To synthesize these peptidyl imaging agents, the carboxylates of DOTA have been conjugated to the amines of peptides, including the N-terminus, the side chain of lysine, and unnatural amino acid derivatives such as p-NH$_2$-phenylalanine.\textsuperscript{21} Other DOTA derivatives have been devised for conjugation to peptide amino groups, such as succinimide DOTA derivatives\textsuperscript{22} and isothiocyanate DOTA derivatives.\textsuperscript{23} However,
coupling DOTA only to peptide amines can limit synthesis methodologies.\textsuperscript{24,25} Also, modification of a peptide N-terminus or side chain amino group can compromise the utility of the peptide for molecular imaging applications, because peptide-biomolecule interactions critically depend on the (native) peptide amino acid sequence.

To address these limitations, a facile methodology is required for conjugating DOTA to peptide carboxylates, especially the C-terminus of a peptide. We have developed new $\alpha$-amino-DOTA derivatives to conjugate directly to the C-terminus of a peptide and used two different glycine templates (BOC and CBZ protected) to accommodate future applications via orthogonal protection strategies. BOC protection is more advantageous in solution phase synthesis, and CBZ protection has advantages in orthogonal synthesis and more complex structures. We have conjugated these DOTA derivatives to lanthanide ions. Finally, we have confirmed that $\alpha$-amino-DOTA-Gd$^{3+}$ has good $T_1$ MR relaxivity to demonstrate that these DOTA derivatives may serve as molecular imaging agents.
2. Experimental Methods

A. General Methods

All the reactions were carried out under argon atmosphere. Dichloromethane and carbon tetrachloride were freshly distilled over P₂O₅. Acetonitrile was distilled over BaO. Fmoc-protected amino acids, acetic anhydride, HBTU (O-Benzotriazol-1-yl-N,N,N’N’-tetramethyluronium hexafluorophosphate), HOBt (1-Hydroxybenzotriazole hydrate), piperidine, DIEA (N,N-diisopropylethylamine) and solvents for peptide synthesis were purchased from Applied Biosystems Co. The DO3A-tBu was purchased from Macrocyclics Co., and other reagents were purchased from Aldrich and Fisher Scientific. Peptides were synthesized using Applied Biosystems 433A Peptide synthesizer using Wang resin (Fluka) and followed Fmoc-chemistry with HBTU and HOBt as coupling agents. The coupling efficiency of each amino acid residue was checked by conductivity installed in the peptide synthesizer. The resin has 0.75 mmol/g of OH groups and the scale of peptide synthesis was calculated based on the substitution ratio of the resin. To purify the peptides and the peptidyl-aminoDOTA final product, a HPLC and fraction collector were used with a C-18 reverse phase column. For the bromination of Boc-Gly-tBu, a 4500μW/cm² UV lamp was used with a filter, and a water bath was used to maintain the temperature of reaction solution at 20°C. The lanthanide complexation
reaction between Peptidyl-aminoDOTA and GdCl₃ was evaluated with an Arsenazo III optical dye test that detects free lanthanides. ¹H and ¹³C spectra were recorded on a Varian Gemini 300MHz NMR spectrometer for ¹H NMR and 600MHz NMR spectrometer for ¹³C NMR (300 and 125 MHz, respectively) using CDCl₃ and DMSO-d₆ as solvents depending on solubility. Analytical thin layer chromatography was performed on Merck silica gel 60 F254 plates. Compounds were visualized using a UV lamp (254nm), iodine chamber and ninhydrin color test. High resolution mass spectral analyses were performed with Mass Spectrometer (Bruker Daltonics Esquire HCT) and MALDI-MASS spectrometer (Bruker BIFLEX III MALDI-TOF).

B. Synthesis and characterization

i) N-(Benzyloxycarbonyl)-α-hydroxyglycine (CBZ-Gly(OH)-OH)

Benzylcarbamate (15.1g, 0.1mol) and glyoxylic acid monohydrate (9.2g, 0.1mol) were stirred together for 6 days in dry diethylether (250 mL). The initial solution appeared as a suspension, but the solution become clear within 6 hrs and the product was precipitated as white solid after 2 days. The product was filtered and used crude for further reactions. White solid (21.5g, quantitative yield): ¹H NMR (300MHz, CDCl₃) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H); ¹³C (125MHz, DMSO-d₆) δ 172.77, 156.17, 137.49,
ii) Methyl N-(Benzylxycarbonyl)-α-methoxyglycinate (CBZ-Gly(OMe)-OMe)

Concentrated sulfuric acid (1.0 mL) was added to an ice-cooled solution of N-(Benzylxycarbonyl)-α-hydroxyglycine (6.0g, 26.6mmol) in anhydrous methanol (100 mL). The reaction was allowed to warm to room temperature and stirred for 48hrs. At this point the reaction was quenched by being poured into ice-saturated NaHCO₃ (saturated aqueous, 200 mL). The product was extracted into EtOAc (4 X 100 mL) and the organic phase was dried over anhydrous MgSO₄, filtered and concentrated in vacuo, yielding product as a powdery white solid (95%). The product was obtained with quantitative yield and used without further purification: ¹H NMR (300MHz, CDCl₃) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H); ¹³C (125MHz, DMSO-d₆) δ 168.60, 156.55, 137.23, 129.07, 128.64, 128.55, 81.03, 66.56, 55.37, 52.85; MS-ESI m/z: 256.21(calcd. 253.10) [M+H]+

iii) Methyl N-(Benzylxycarbonyl)-α-bromoglycinate (CBZ-Gly(Br)-OMe)

Phosphorous tribromide (8.2g, 30mmol, 3eq.) was added to a suspension of methyl N-(Benzylxycarbonyl)-α-methoxyglycinate (2.51g, 10mmol) in carbon tetrachloride (100
mL), under an argon atmosphere. The reaction mixture was stirred at room temperature for 7 days. At this point the reaction solution was concentrated in vacuo and triturated with dry n-hexane (100 mL) for 24 hrs. The reaction mixture was then filtered, yielding a white solid (75% to quantitative yield): \(^1\)H NMR (300MHz, CDCl\(_3\)) \(\delta\) 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H); \(^{13}\)C (125MHz, DMSO-d\(_6\)) \(\delta\) 170.71, 156.07, 137.37, 129.03, 128.57, 128.53, 73.78, 66.25; MS-ESI \(m/z\): 304.21 (calcd. 302.12) [M+H]^+

iv) \textit{t-Butyl 2-Bromo-2-(t-butoxycarbonylamino)acetate (Boc-Gly(Br)-tBu)}

N-bromosuccinimide (1.78g, 10mmol) was added to a solution of Boc-Gly-OtBu (2.31g, 10mmol) in dry carbon tetrachloride (50 mL) and the mixture was irradiated with an UV lamp at 20°C for 1.5hrs. The succinimide was filtered off and the filtrate was concentrated in vacuo. The residual oil phase was crystallized on drying. The product was obtained in quantitative yield (3.00g, 97%) and used without further purification: \(^1\)H NMR (300MHz, CDCl\(_3\)) \(\delta\) 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H); \(^{13}\)C (125MHz, DMSO-d\(_6\)) \(\delta\) 171.01, 156.29, 79.51, 63.72, 28.53; MS-ESI \(m/z\): 304.22 (calcd. 309.06) [M+H]^+
v) *Methyl N-(Benzyloxycarbonyl)-DO3A(tBu)-glycinate (CBZ-Gly(DO3A-tBu)-OMe)*

Methyl N-(Benzyloxycarbonyl)-α-bromoglycinate (0.6g, 2mmol) was dissolved in dry acetonitrile (100 mL) with DO3A(tBu) (1.0g, 1mmol) in the presence of K₂CO₃ (1.66g, 12mmol, 6eq.). The reaction mixture was heated to 70°C for 6hrs. After removal of the undissolved solids by filtration, the solution was concentrated in vacuo, yielding product as a powdery solid (90%): ¹H NMR (300MHz, CDCl₃) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H); ¹³C (125MHz, DMSO-d₆) δ 171.43, 170.44, 170.10, 156.53, 136.71, 129.68, 128.22, 127.64, 79.06, 78.85, 78.63, 69.72, 65.51, 64.81, 57.96, 56.20, 55.75, 55.56, 51.89, 51.37, 49.76, 49.31, 48.35, 47.72, 47.39, 45.80, 27.75; MS-ESI m/z: 738.73 (calcd. 735.91) [M+H]⁺

vi) *t-Butyl 2-DO3A(tBu)-2-(t-butoxycarbonylamino)acetate (Boc-Gly(DO3A-tBu)-tBu)*

Boc-Gly(Br)-tBu (0.66g, 2.2mmol) was dissolved in dry acetonitrile (100 mL) with DO3A(tBu) (1.0g, 2mmol) in the presence of K₂CO₃ (1.66g, 12mmol, 6eq.). The reaction mixture was heated to 70°C for 6hrs. After removal of the undissolved solids by filtration, the solution was concentrated in vacuo, yielding product as a powdery solid (90%): ¹H NMR (300MHz, CDCl₃) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H); ¹³C (125MHz, DMSO-d₆) δ 171.45, 171.43, 171.37, 80.78, 80.65, 77.61, 57.45, 51.96,
vii) Amino DOTA from CBZ-Gly(DO3A-tBu)-OMe

CBZ-Gly(DO3A-tBu)-OMe (1.3g, 2mmol) was dissolved in 50% TFA in dry dichloromethane (50 mL). The deprotection reaction mixture was stirred for 12 hrs at room temperature. After removal of the half of solvents under reduced pressure, the product was precipitated by addition of diethylether. The product was filtered and dried in vacuo. To purify the product, it was recrystallized in dichloromethane-diethylether, the precipitated product was filtered and dried in vacuo, yielding product as white solid (90%): ¹H NMR (300MHz, CDCl₃) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H); ¹³C (125MHz, DMSO-d₆) δ 173.44, 172.87, 78.52, 53.33, 52.14, 47.93; MS-ESI m/z: 413.24 (calcd. 419.43) [M+H]⁺

viii) Amino DOTA from Boc-Gly(DO3A-tBu)-tBu

Boc-Gly(DO3A-tBu)-tBu (1.3g, 2mmol) was dissolved in 50% TFA in dry dichloromethane (50 mL). The deprotection reaction mixture was stirred for 6 hrs at room temperature. After removal of half of the solvents under reduced pressure, the product was precipitated by addition of diethylether. The product was filtered and dried in vacuo.
To purify the product, it was recrystallized in dichloromethane-diethylether, and the precipitated product was filtered and dried in vacuo, yielding product as white solid (90%): $^1$H NMR (300MHz, CDCl$_3$) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H); $^{13}$C (125MHz, DMSO-d$_6$) δ 173.44, 172.87, 78.52, 53.33, 52.14, 47.93; MS-ESI $m/z$: 413.34 (calcd. 419.43) [M+H]$^+$

ix) **Arsenazo III color test**

The arsenazo III disodium salt was dissolved in acetate buffer solution at pH 4 and a concentration of 10 μM. A small aliquot of the complexation reaction mixture was tested for free Gd$^{3+}$ when added to 1.0 mL of 10 μM arsenazo III in acetate buffer. When there are free Gd$^{3+}$ ions, the color test will show blue and the test was determined as positive. When there’s no color change, the test was determined as negative and the complexation was stopped.

x) **Peptide synthesis (Ac-PLGMWSG-OH)**

The peptide was synthesized using an automated peptide synthesizer (Applied Biosystems 433A Peptide synthesizer). The Wang resin was used as a polymer support and the amino acid sequence was Ac-Pro-Leu-Gly-Met-Trp-Ser-Gly-OH. At every
The coupling efficiencies were evaluated with a conductivity measurement that is automatically performed by the synthesizer. The synthesized peptides were cleaved from resin with the treatment condition of TFA/thioanisole/water (95%/1%/4%) cocktail for 30 min. The resin was filtrated and washed with dichloromethane (3 X 30 mL). The filtrate was concentrated in vacuo, and when a small portion of solution was remained, the peptide was precipitated with addition of diethylether. The precipitated peptide was filtered and dried in vacuo. For further purification, the peptide was dissolved in dichloromethane and purified with HPLC with a reverse phase column. The purified peptide was collected and freeze-dried, yielding product as a white solid: MALDI-Mass 789.92 m/z: (calcd. 788.91) [M+H]^+, 811.79[M+Na]^+

xi) Complexation of Gd^{3+} with α-aminoDOTA

The α-amino DOTA (84mg, 0.2mmol) was dissolved in water at pH 6 and 60°C, and GdCl₃ (55.0mg, 0.2mmol) was added to this solution and stirred for 1 hr. With 1N-NaOH solution, the pH was adjusted to 8 and stirred for 30 min. The complexation with lanthanide was checked with the Arsenazo III color test. When the test showed a negative result, the reaction mixture was cooled to room temperature. The solution was freeze dried, yielding a quantitative product: MALDI-Mass m/z: 589.37 (calcd. 588.36) [M+H]^+
Amino DOTA-peptide conjugate

The peptide (236.7 mg, 0.3 mmol) was dissolved in NMP (30 mL) with 227.6 mg of HBTU and 91.9 mg of HOBr for 40 min to activate the carboxyl group of peptide. The α-amino DOTA (125.8 mg, 0.3 mmol) was added for 30 min at room temperature. The reaction mixture was stirred for 1 hr at room temperature. After removal of half of the solvents under reduced pressure, the product was precipitated by addition of diethylether. The product was filtered and dried in vacuo. The obtained peptide was completely dried, yielding product as a white solid: MALDI-Mass 1190.3 m/z: (calcd. 1190.33) [M+H]^+
3. Results and Discussions

We have developed new $\alpha$-amino-DOTA derivatives to conjugate directly to the C-terminus of a peptide, and we have used two different glycine templates (BOC and CBZ protected) to accommodate future applications via orthogonal protection strategies. BOC protection is more advantageous in solution phase synthesis, and CBZ protection has advantages in orthogonal synthesis and more complex structures. We have also chelated lanthanide ions with these DOTA derivatives. Finally, we have confirmed that $\alpha$-amino-DOTA-Gd$^{3+}$ has good $T_1$ MR relaxivity to demonstrate that these DOTA derivatives may serve as molecular imaging agents.

Two different synthetic pathways for the $\alpha$-bromination of glycine templates are shown in Scheme 3.1. The compound 2$^{26}$ was obtained by following previous reported methods that use N-bromosuccinimide and filtered UV radiation (254nm). The bromination showed high efficiency in purity as determined by NMR.$^{27-30}$ The compounds 5~7$^{31}$ were synthesized according to a previously reported synthetic pathway$^{32}$ and acquired with overall yield of 70% as determined by weight.

The compound 9$^{33}$ was synthesized from 2 and 8 and purified with a silica column. To continue further experiments (Scheme 3.2), the protecting groups of 9 were completely removed with a cleavage cocktail (95% TFA/2.5% water/2.5% thioanisole)
for 30 minutes. The solution was concentrated in vacuo and precipitated with ice-cooled diethyl ether and purified with an amberlite column, yielding 11 as white solid.34

To prepare 10,35 the compound 7 was coupled to 8 using the exhaustive alkylation conditions and purified with a silica column. To remove the CBZ group by hydrogenolysis, 10 was dissolved in 10mL of absolute ethanol, and 1,4-hexadiene (0.94 mL, 10mmol) and 10% Pd/C (0.55g, 10mmol) were added to the solution. The remaining methyl and tert-butyl protecting groups were removed by hydrolysis using 1N-NaOH solution. The aqueous solution was lyophilized after purification with an amberlite column, yielding a slightly yellowish solid of 11.

Due to the bulky protecting groups of 2 and 7, the reaction yields were expected to be lower than conventional exhaustive alkylation reactions, but the reactions showed sufficiently high yield to be used in subsequent synthesis steps without further purification. This demonstrates the facile nature of the synthesis methodology. The α-bromo glycine templates were produced as racemates so that (R)- and (S)- types were formed at approximately a 1 to 1 ratio, as evidenced by doublets at 5.20 and 5.46 ppm in the NMR spectrum. After the α-bromo glycine templates were coupled to 8, the diastereomers in 9 and 10 were also determined to be the same ratio as the α-bromo glycine templates.
To demonstrate the coupling of 11 with the C-terminus of a peptide, a peptide was synthesized with a Wang resin (Fluka, 0.75mmol/g), HBTU and HOBT coupling agents, and standard Fmoc chemistry protocols using an Applied Biosystems 433A peptide synthesizer. The synthesized peptide sequence was selected to be a substrate for the MMP-2 enzyme. The MMP-2 targeting peptide sequence was Ac-Pro-Leu-Gly-Met-Trp-Ser-Gly (Ac-PLG-MWSG). The peptide was cleaved from the resin with a 95% TFA/ 2.5% water/ 2.5% thioanisole cocktail for 30 minutes. The peptide was purified by crystallization in dichloromethane/diethyl ether and an amberlite column (yield 90% by weight) and characterized with a MALDI mass spectrometer (m/z: 811.90 (calcd. 811.91) [M+Na]+).

To show the versatile application of coupling 11 to the C-terminus of a peptide to synthesize peptidyl-DOTA structure 13, the synthesized peptide (120mg, 0.15mmol) was dissolved in NMP (5 mL) with 63mg of HBTU and 26mg of HOBT and stirred for 40 minutes to activate the carboxyl group of the peptide. 11 (80mg, 0.15mmol) and TEA (110 µL, 0.75mmol) in 1 mL of NMP was added slowly at room temperature. The reaction mixture was stirred for 1hr. After removal of half of the solvents under reduced pressure, the product 13 was precipitated by addition of diethylether (100 mL). The obtained crude product was purified with an amberlite column, yielding product as a
white solid (yield 71% by weight) and characterized with a MALDI-MASS spectrometer $(m/z: \text{1190.3 (calcd. 1189.54) [M+H]}^+)$.

To verify that 11 can serve as a molecular imaging contrast agent, the $T_1$ relaxivity of 12 was measured to assess the efficiency of the chelate to alter $T_1$-weighted MR image contrast. 11 (25.2mg, 0.06mmol) was dissolved in water at pH 6 and 60°C, and GdCl$_3$ (150 μL of 0.4 mM solution) was added to this solution and stirred for 1 hr. The pH was adjusted to 8 using 1N NaOH and stirred for 48hrs. The chelation was monitored using a standard Arsenazo III color test.$^{41}$ $T_1$ measurements of 12 were conducted at 18°C and pH 7.1, with samples ranging from 5 to 50 mM. The $T_1$ inversion-recovery experiment was conducted with a 600 MHz NMR spectrometer. The measured relaxivity of 4.83 mM$^{-1}$ sec$^{-1}$ compared very favorably to the 3.83 mM$^{-1}$ sec$^{-1}$ relaxivity of Gd$^{3+}$-DOTA that is used in clinical MRI studies.$^{42}$
4. Conclusions

This report has demonstrated the preparation and application of newly synthesized chelators for lanthanides, 11 ~ 13. Also, compound 11 was synthesized from two different glycine templates to accommodate future solution phase and solid phase synthesis methods. The incorporation of an amino group facilitated the conjugation of 11 to the C-terminus of a peptide backbone with high efficiency and relatively high yield. Coupling of 11 to peptide carboxylates greatly expands peptide-DOTA synthesis strategies by complementing standard methods that couple DOTA to peptide amines. Furthermore, the number of amino groups incorporated into DOTA can be controlled from 1 to 4 by the selection of the macrocyclic starting material, such as cyclen (1,4,7,10-tetraazacyclododecane), DO1A-p-Bu, DO2A-p-Bu or DO3A-p-Bu.
5. References


26. N-bromosuccinimide (1.78g, 10mmol) was added to a solution of 1 (2.31g, 10mmol) dissolved in dry carbon tetrachloride (50mL). The mixture was irradiated with a 254nm filtered UV lamp at 25°C for 1.5hrs (3.00g, yield 97%). ¹H NMR (300MHz, CDCl₃) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H); ¹³C (125MHz, DMSO-d₆) δ 171.01, 156.29, 79.51, 63.72, 28.53; MS-ESI m/z: 310.02 (calc. 309.06) [M+H]⁺.
31. ¹H NMR (300MHz, CDCl₃) δ 3.80(s, 3H), 5.22(s, 2H), 6.20 and 6.45(split, 1H), 7.55(s,5H); ¹³C (125MHz, DMSO-d₆) δ 170.71, 156.07, 137.37, 129.03, 128.57, 128.53, 73.78, 66.25; MS-ESI m/z: 304.07 (calc. 302.99) [M+H]⁺.
33. 2 (0.62g, 2.0mmol) in dry acetonitrile (100mL) was stirred with 8 (1.1g, 2.1mmol) in the presence of K₂CO₃ (1.66g, 12mmol, 6eq.). The reaction mixture was heated to 70°C for 6hrs. After removal of the undissolved solids by filtration, the solution was concentrated in vacuo. The product was purified with silica column using ethylacetate as an eluent, yielding product as a powdery solid (yield 95%). ¹H NMR (300MHz, CDCl₃) δ 1.45(s, 45H), 2,76(t, 16H), 3.40(s, 6H), 5.20 and 5.46(split, 1H); ¹³C (125MHz, DMSO-d₆) δ 171.45, 171.43, 171.37, 80.78, 80.65, 77.61, 57.45, 51.96, 46.93, 28.46, 28.30; MS-ESI m/z: 744.44 (calc. 743.50) [M+H]⁺.
34. Yield 90%. $^1$H NMR (300MHz, CDCl$_3$) δ 2.0(s, 2H), 2.80(t, 16H), 3.60(s, 6H), 5.20 and 5.46(split, 1H); $^{13}$C (125MHz, DMSO-d$_6$) δ 173.44, 172.87, 78.52, 53.33, 52.14, 47.93; MS-ESI $m/z$: 459.31 (calc. 458.17) [M+K]$^+$. 

35. 7 (0.6g, 2mmol) was coupled to 8 (1.1g, 2.1mmol) using the exhaustive alkylation conditions and the product was purified with silica column using ethyl acetate as an eluent (yield 90%). $^1$H NMR (300MHz, CDCl$_3$) δ 1.45(s, 27H), 2.78(t, 16H), 3.40(s, 6H), 3.80(s, 3H), 5.20 and 5.46(split, 1H), 7.20(s, 5H); $^{13}$C (125MHz, DMSO-d$_6$) δ 171.43, 170.44, 170.10, 156.53, 136.71, 129.68, 128.22, 127.64, 79.06, 78.85, 78.63, 69.72, 65.51, 64.81, 57.96, 56.20, 55.75, 55.56, 51.89, 51.37, 49.76, 49.31, 48.35, 47.72, 47.39, 45.80, 27.75; MS-ESI $m/z$: 736.34 (calc. 735.44) [M+H]$^+$. 


Scheme 3.1 Synthesis of α-brominated glycine templates: (i) N-bromosuccinimide, UV (254nm, filtered), CCl₄, 25°C, 1.5hrs, 97%; (ii) diethylether, rt, 6days, 96%; (iii) MeOH, H₂SO₄, rt, 2days, 95%; (iv) PBr₃, CCl₄, rt, 7days, 75%.
Scheme 3.2 Synthesis of α-amino-DOTA derivatives and coupling to a peptide backbone carboxylate group. (i) K₂CO₃ (6eq.), acetonitrile, 70°C, 6hrs, 95%; (ii) K₂CO₃ (6eq.), acetonitrile, 70°C, 6hrs, 90%; (iii) 95% TFA/2.5% water/2.5% thioanisole, 30min, 95%; (iv) 1,4-cyclohexadiene/10% Pd-C, EtOH; (v) 1N-NaOH; (vi) amberlite column; 71%; (vii) pH, 60°C, GdCl₃ (150ul of 0.4mM solution), 48hrs. (viii) Ac-PLGMWSG-OH (1eq.), HBTU (1.1eq.), HOBt (1.1eq.), TEA (5eq.), NMP, rt, 1hr; (viii) dialysis (MWCO-100); total yield 85% by weight.
Figure 3.1 $^1$H-NMR spectrum of Cbz-Gly(OH)-OH

Figure 3.2 $^1$H-NMR spectrum of Cbz-Gly(OMe)-OMe
Figure 3.3 $^1$H-NMR spectrum of Cbz-Gly(Br)-OMe

Figure 3.4 $^1$H-NMR spectrum of Boc-Gly(Br)-tBu
Figure 3.5 $^1$H-NMR spectrum of Boc-Gly-tBu-DO3A-tBu (Boc-amino-DOTA)

Figure 3.6 $^1$H-NMR spectrum of Cbz-Gly-OMe-DO3A-tBu (Cbz-amino-DOTA)
**Figure 3.7** $^1$H-NMR spectrum of amino-DOTA

**Figure 3.8** Maldi-Mass spectral analysis of Ac-PLGMWSG-amino-DOTA.
Chapter IV. Solid Phase Synthesis of Molecular Imaging Contrast Agents

Byunghee Yoo and Mark D. Pagel
Peptidyl Molecular Imaging Contrast Agents
Using a New Solid-Phase Peptide Synthesis Approach
*Bioconjugate Chemistry* 2007, 18, 903-911.
1. Introduction

Macrocyclic metal chelates using DOTA (1,4,7,10-tetraazacyclododecane-
$N,N',N'',N'''$-tetraacetic acid) are often administered to patients and animal models to
create or enhance contrast in biomedical molecular imaging studies.$^{1,2}$ More recently,
metal-DOTA chelates have been conjugated to peptides to affect the in vivo
pharmacokinetics of the metal-DOTA imaging agent over a wide range of spatial scales.
For example, sub-cellular scales can be assessed by coupling DOTA to membrane-
penetrating peptides,$^{3,4}$ cellular scales are assessed with cell receptor-targeting peptides
attached to DOTA,$^{5}$ and tissue scales are investigated with DOTA-bound peptides that
interact with extracellular matrices$^{6}$ and that have dramatically altered renal clearance
rates.$^{7}$ Numerous other applications have been reported, including peptide-DOTA
probes for multimodality imaging studies.$^{8-10}$

To synthesize these peptidyl molecular imaging contrast agents, the carboxylates of
DOTA have been conjugated to the amines of peptides, including the N-terminus of the
backbone and the side chain of lysine.$^{11-19}$ Other DOTA derivatives have been devised
for conjugation to peptide amino groups, such as succinimide DOTA derivatives$^{20}$ and
isothiocyanato DOTA derivatives.$^{21}$ Unnatural amino acid derivatives have also been
developed to couple DOTA to peptidyl amines, such as p-NH₂-phenylalanine\textsuperscript{13} and
diaminopropionic acid residues.\textsuperscript{15}

Standard solid phase peptide synthesis (SPPS) methods have been used to couple
DOTA to the backbone N-terminus of peptides bound to a PEGA Rink amide resin.\textsuperscript{5,14-19}

Similar SPPS methodologies can be used to couple DOTA to other side chain amines of
resin-bound peptides.\textsuperscript{13,15} However, coupling DOTA only to N-terminus or side chain
amines of peptides can limit synthesis methodologies and may compromise the utility of
the peptidyl contrast agent for molecular imaging applications.\textsuperscript{22} To alleviate this
limitation, we have developed an amine-derivatized DOTA (aminoDOTA) that can
couple to the carboxylates of peptides (Scheme 4.1). We have linked the aminoDOTA
to a resin \textsuperscript{19a} for use in standard SPPS methodologies, in order to synthesize peptide-
DOTA imaging agents with DOTA coupled to the C-terminus of the peptide (Scheme
4.2). We have also linked aminoDOTA to an amino acid residue on the resin \textsuperscript{19b}, to
demonstrate that this methodology can incorporate DOTA at any location within the
peptide backbone (Schemes 4.1 and 4.2). Lanthanide chelates of the peptide-DOTA
amide products were shown to affect magnetic resonance signals, thereby demonstrating
that the peptide-bound DOTA moiety has practical utility for molecular imaging. This
new approach greatly expands the opportunities to create peptide-based molecular
imaging probes, and is completely compatible with standard SPPS methods for facile production of products with good yield and high purity.
2. Experimental Methods

A. General Methods

All the reactions were carried out under argon atmosphere. Dichloromethane (DCM) and carbon tetrachloride (CCl₄) were freshly distilled over phosphorous pentoxide (P₂O₅). Acetonitrile (ACN) was distilled over barium oxide (BaO). The peptide synthesis reagents Fluorenylmethyloxycarbonyl (Fmoc) protected amino acids, N-hydroxybenzotriazole (HOBT), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylyluronium hexafluorophosphate (HBTU), acetic anhydride (Ac₂O), piperidine, triethylamine (TEA), N,N-diisopropylethylamine (DIEA), N,N-dimethylformamide (DMF) and N-methylpyrrolidone (NMP) were purchased from Applied Biosystems Co. (Foster City, CA). The 1,4,7,10-tetraazacyclododecane-Ν,Ν′,Ν″-tri(tert-butylacetate) (DO3A-tBu) was purchased from Macrocyclics Co. (Dallas, TX), and other reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and Fisher Scientific International, Inc. (Hampton, NH). Peptides were synthesized using a Wang resin (Fluka, Buchs Switzerland) and followed Fmoc chemistry methods with HOBT and HBTU as coupling agents. The efficiencies of coupling DO3A-Fmoc 19 to resin 16b, and coupling 2 to resin Fmoc-deprotected 17a and 17b, were checked with the Kaiser ninhydrin test. The starting resin had 1.0mmol/g of hydroxyl groups or amino groups and the scale of the
peptide synthesis was calculated based on the substitution ratio of the resin. The loading efficiencies of the Fmoc-compounds and the amine content of the resin were quantitatively analyzed with UV/Vis/Fluorescence spectroscopy using a Molecular Devices SpectraMax M2 spectrometer. FT-IR spectroscopy was used to analyze the functional groups on the resin using an ABB BOMEM MB-104 spectrometer operating from 600 to 4000 cm\(^{-1}\). To analyze the peptides and the peptide-DOTA amide final product, HPLC was used with a Grace Vydac OD-300 C-18 reverse phase analytical column and a PerkinElmer Series 200 HPLC pump and UV detector operating at 222 nm. The lanthanide complexation reaction between peptide-DOTA amide and TmCl\(_3\) was evaluated with an Arsenazo III solution color test.\(^{28,29}\) Analytical thin layer chromatography was performed on Merck silica gel 60 F254 plates. Compounds were visualized using a UV lamp operating at 254 nm, an iodine chamber and ninhydrin solution. High resolution mass spectral analyses were performed with Bruker Daltonics Esquire HCT mass spectrometer and a Bruker BIFLEX III MALDI-TOF mass spectrometer, using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix compound. Molecular modeling was accomplished using InsightII with the Discover-3 (Molecular Simulations Inc.). \(^1\)H and \(^{13}\)C spectra were measured with a Varian Gemini 300MHz NMR spectrometer and
Varian Inova 600MHz NMR spectrometer using CDCl₃ and DMSO-d₆ as solvents depending on solubility.

PARAmagnetic Chemical Exchange Saturation Transfer (PARACEST) spectra were measured in a solution of 5% D₂O in water¹⁰,³¹ using a Varian Inova 600 MHz NMR spectrometer with a modified presaturation pulse sequence that included a continuous wave saturation pulse, saturation pulse power of 31 μT, saturation delay of 4 seconds and in 1 ppm increments from 100 to -100 ppm. MR images were acquired with a Bruker Biopsin 9.4 T MR scanner. A MSME T₁ method was used with TR/TE = 1623/10.9 ms and a train of Gaussian-shaped saturation pulses applied at 50 μT for 1.106 s, and with saturation offsets at -51 and +51 ppm.

**B. Synthesis and characterization**

i) 4,7,10-tri(t-butylacetate)-1-fluorenymethoxycarbonyl-1,4,7,10-tetraazacyclo-
dodecane (Fmoc-DO₃A-tBu) 14

To a solution of 8 (2.57g, 5mmol) in 10 mL of acetonitrile, activated zinc dust was added in small portions until the reaction mixture attained neutral pH.³² A solution of Fmoc-
Cl (1.35g, 5mmol, 1 eq.) in 5 mL of ACN and zinc dust (325mg, 5mmol, 1 eq.) was added to the reaction mixture in one portion and the reaction mixture was stirred at room
temperature for 20 min. The progress of the reaction was monitored by TLC (CHCl₃/MeOH=1/1, Rf=0.75). The reaction mixture was filtered and dried in vacuo, yielding 3.32g of white solid (yield 90%): ¹H NMR (600MHz, DMSO-d₆) δ 1.47(s, 27H), 2.50(s, 8H), 2.71(t, 4H), 2.79(t, 4H), 3.39(s, 6H), 5.15(t, 1H), 6.29(d, 2H), 7.42(t, 2H), 7.47(t, 2H), 7.63(d, 2H), 7.84(d, 2H), ¹³C (125MHz, DMSO-d₆) δ 170.61, 153.83, 135.59, 135.36, 129.47, 127.23, 123.88, 121.36, 119.99, 81.22, 65.88, 55.14, 53.53, 50.48, 48.44, 47.10, 27.75, MALDI-Mass m/z (calc. 736.94): 737.96 [M+H]⁺.

ii) 4,7,10-tri(carboxymethyl)-1-fluorenymethoxycarbonyl-1,4,7,10-tetraazacyclo-dodecane (Fmoc-DO3A) 15

A total of 2.94g (4mmol) of 14 was dissolved in 5 mL of 75% TFA in DCM and treated for 40 min. The reaction was traced by TLC (chloroform/methanol=1.1, Rf=0.22). The solution was dried under reduced pressure. The remaining solid was re-dissolved in DCM and precipitated with diethyl ether. The precipitated solid was filtered and dried in vacuo, yielding 1.70g (3.0mmol, yield 75%): ¹H NMR (600MHz, DMSO-d₆) δ 3.05(s, 4H), 3.20(s, 4H), 3.45(t, 4H), 3.62(t, 4H), 3.60(s, 6H), 4.25(t, 1H), 4.55(d, 2H), 7.35(t, 2H), 7.42(t, 2H), 7.65(d, 2H), 7.94(d, 2H), ¹³C (125MHz, DMSO-d₆) δ 174.21, 155.44, 143.85,
iii) Coupling of Fmoc-DO3A on the resin 17a and 17b

To prepare 5a, 1.0 g of Wang resin 16a (substitution level = 1 mmol/g) was used after complete drying. The compound 15 (1.14g, 2mmol), HBTU (2.5g, 6.6mmol, 3.3 eq.) and HOBt (1.0g, 6.6mmol, 3.3 eq.) were dissolved in 30 mL of NMP for 40 min to activate carboxylates, and the solution was added to the peptide reaction vessel containing the resin. TEA (1.82 mL, 13 eq.) was added and the reaction was continued for 12 hrs. After filtration, the resin was dispersed in a solution of tert-butyl alcohol (t-BuOH, 1.48g, 20mmol, 20 eq.) in 20 mL of NMP and the reaction was continued for 1 hr. The resin was washed and dried in vacuo. To prepare 17b, 1.3g of 16b (substitution level = 0.78 mmol/g) was treated with 15 following the same synthetic method as 16a. During the reaction, a small amount of resin was sampled for uncoupled amines using the Kaiser test.
iv) **Loading Efficiency (Fmoc titration)**

The Fmoc concentration on the resin was measured according to a previously reported method.\textsuperscript{33} Fmoc amino acyl resins (4~8mg) were shaken or stirred in piperidine-DMF (3:7) (0.5 mL) for 30 min, after which MeOH (6.5 mL) was added and the resin was allowed to settle. The resultant fulvene-piperidine adduct had UV absorption maxima at 267 nm (ε = 17,500 M\textsuperscript{-1}cm\textsuperscript{-1}), 290 nm (ε = 5800 M\textsuperscript{-1}cm\textsuperscript{-1}), and 301 nm (ε = 7800 M\textsuperscript{-1}cm\textsuperscript{-1}). For reference, a piperidine-DMF-MeOH solution (0.3:0.7:39) was prepared. Spectrophotometric analysis was carried out at 301 nm, with comparison to a free Fmoc amino acid (Fmoc-Ala) of known concentration treated under identical conditions. The measured Fmoc concentrations were determined to be 0.45 (± 0.05) mmol per gram of resin (calc. 0.64 mmol/g) for 17a and 0.47 (± 0.05) mmol per gram of resin (calc. 0.54 mmol/g) for 17b, based on 3 repetitions to synthesize each product.

Sub. Level = Abs. × 10\textsuperscript{6}μmol/mol × 0.007L × 7800 × 1cm × mg of resin

v) **Methyl N-(Benzyloxycarbonyl)-α-bromoglycinate 2**

Phosphorous tribromide (8.2g, 30mmol, 3 eq.) was added to a suspension of methyl N-(Benzyloxycarbonyl)-α-methoxyglycinate (2.51g, 10mmol) in carbon tetrachloride (100
mL) under an argon atmosphere. The reaction mixture was stirred at room temperature for 7 days. The reaction solution was then concentrated in vacuo and triturated with dry n-hexane (100 mL) for 24 hrs. The reaction mixture was then filtered, yielding a white solid (75% to quantitative yield measured by weight): $^1$H NMR (300MHz, CDCl$_3$) $\delta$ 3.80(s, 3H), 5.22(s, 2H), 6.20 and 6.45(split, 1H), 7.55(s, 5H); $^{13}$C (125MHz, DMSO-d$_6$) $\delta$ 170.71, 156.07, 137.37, 129.03, 128.57, 128.53, 73.78, 66.25; MS-ESI $m/z$ (calc. 302.99): 304.07 [M+H]$^+$

vi) **Coupling of 2 on the resin 18a and 18b**

Each of 17a (2.22g, substitution level =0.45 mmol/g) and 17b (2.13g, substitution level =0.47 mmol/g) were treated with 20% piperidine for 30 min and sequentially washed with NMP, DCM, acetone, and acetonitrile. The Kaiser test was used to measure the content of free secondary amines after Fmoc deprotection. The resin was transferred to a flask with 2 (0.6g, 2mmol, 4.3 eq.) and K$_2$CO$_3$ (1.66g, 12mmol, 25.5 eq.) in dry acetonitrile (100 mL). The solution was stirred and heated to 70 °C for 6 hrs in anhydrous conditions. After the solution was filtered, the resin was sequentially washed with 50% MeOH in water, MeOH, and DCM, and then dried in vacuo. The Kaiser test was used
to monitor the coupling of 2 to \textbf{18b} by measuring the concentration of remaining free amines.

\textit{vii) Cleavage of CBZ group from the resin \textbf{19a} and \textbf{19b}}

In each of 3 flasks, 0.20g (0.1mmol) of \textbf{18a} was dispersed and swelled in DCM (5 mL) for 1 h and cooled to -78 °C. Et\textsubscript{2}AlCl\textsuperscript{34} was transferred into each flask with an air-tight syringe in 1, 2 and 5 eq. and the mixture was stirred for 15 min. Aliquots of 2, 4, and 10 eq. of thioanisole (the molar ratio of Et\textsubscript{2}AlCl:thioanisole was fixed at 1:2) was added to each flask and portions of the resin were extracted after 5, 15, 30 and 60 min of cleavage reaction time. The sampled resin was immediately washed with DCM and titrated with picric acid according to a previously reported method.\textsuperscript{35} To account for tertiary amines on the cyclen ring, a CBZ protected resin was treated and compared as a reference. CBZ cleavage was performed with conditions of 1 eq. of 33% Et\textsubscript{2}AlCl in thioanisole for 20 min at -78 °C. The average amine content was 0.31 (±0.05) mmol/g for \textbf{19a} and 0.33 (±0.05) mmol/g for \textbf{19b}, based on 3 repetitions to synthesize each product.
Table 4.1 The amine contents on the resin measured by picric acid titration.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Et&lt;sub&gt;2&lt;/sub&gt;AlCl/thioanisol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1eq.</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
</tr>
<tr>
<td>15</td>
<td>0.46</td>
</tr>
<tr>
<td>30</td>
<td>0.32</td>
</tr>
<tr>
<td>60</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Figure 4.1 A ninhydrin color test was performed to qualitatively analyze the amine content on the resin. After 25 minutes of reaction time, the resin showed darkest color and after 30 minutes of reaction time, the color of resin became weaker. The reaction time was fixed at 25 minutes during subsequent studies.

viii) **IR spectra**

CBZ cleaved aminoDOTA-Wang resin was analyzed with FT-IR to confirm the introduction of functional groups. 3440 cm<sup>-1</sup> (cyclen ring N-H, str), 3014 cm<sup>-1</sup> (aromatic
ix) Peptide (DEVD) loading on the resin 20a and 20b

A total of 1.63g (0.5mmole) of 19a was used to synthesize 20a using standard SPPS methods.36 Fmoc-Asp(OtBu)-OH (0.2g, 0.5mmol), Fmoc-Val-OH (0.17g, 0.5mmol) and Fmoc-Glu(OtBu)-OH (0.23g, 0.5mmol) were used as building amino acids and HBTU (0.19g, 0.5mmol) and HOBT (73mg, 0.5mmol) were used as coupling agents. Freshly distilled TEA (140 μL, 1mmol) was used as a base in 70 mL of NMP. A solution of 20% piperidine in NMP was used to cleave the Fmoc groups on the resin, after which 1 equivalent of an amino acid was reacted with the resin-bound peptide-DOTA amine. This coupling was repeated with a second reaction that was not preceded with Fmoc deprotection, in order to follow a double-coupling SPPS strategy, which yielded 1.94g of 20a (calc. 1.97g, yield 93%). Using the same procedure, 0.42g (0.14mmole) of 19b was treated to synthesize 0.50g of 20b (calc. 0.52g, yield 90%).
x) **Peptide cleavage from the resin and characterization**

After the peptide synthesis, the Fmoc group of \(20a\) (0.55g, 0.2mmole) was removed from the resin and \(21a\) was cleaved from the resin with a 95% TFA/2.5% water/2.5% thioanisole cocktail for 40 min.\(^{38-40}\) After removing solvents, the product was washed with diethylether and dried in vacuo. The final product was purified with an amberlite column, yielding 150mg (0.17mmol, yield 85%). Following the same procedure, 0.29g of \(20b\) (0.1mmole) was used to obtain 87 mg of \(21b\) (92μmole, yield 92%). In both cases, the amberlite column was used to remove impurities and residue from the cleavage cocktail, and was not intended to purify isomeric forms of \(21a\) or \(21b\). The obtained products \(21a\) and \(21b\) were characterized by MALDI-MASS (\(21a\) m/z (calc. 891.88): 892.89 [M+H]\(^+\), 914.89 [M+Na]\(^+\); \(21b\) m/z (calc. 948.93): 949.94 [M+H]\(^+\), 971.95 [M+Na]\(^+\)) and HPLC (Column : Symmetry C-18 3.5um, 4.6 x 75mm, Detector : 222 nm, Flow rate : 0.5 mL/min, Eluent : 0.1% TFA in water / acetonitrile, gradient from 100% / 0% to 90% / 10% for 5 min, 90%/10% for 25 min; retention time = 6.1 min for \(21a\) and \(21b\)).

xi) **Lanthanide Complexation of \(21a\) and \(21b\)**

The obtained \(21a\) (100mg, 0.11mmol) was dissolved in water (3 mL) at pH 6.5 and 40 °C,
and TmCl₃ (27mg, 0.1mmol) in water (0.5 mL) was added drop by drop for 1 hr and adjusted to pH 7.5 with 0.5N NaOH. The solution was stirred for 18 hrs at 40°C and adjusted to pH 7.5 when the pH dropped below 5. The complete complexation was evaluated with an Arsenazo III color test. When the test showed negative results for free lanthanide ions, the reaction mixture was cooled to room temperature. The pH was adjusted to 9 and the residual lanthanide-hydroxide white precipitate was removed by filtration. The solution was freeze dried, yielding product 22a (MALDI-Mass m/z (calc. 1060.81): 1061.83 [M+H]⁺, 1083.85 [M+Na]⁺). 21b was treated with a similar procedure to obtain the final product 22b (MALDI-Mass m/z (calc. 1117.86): 1118.87 [M+H]⁺, 1140.92 [M+Na]⁺). To create a reference for the product of the protease reaction, the same procedure was used to chelate Tm with α-amino-DOTA to form α-amino-(Tm-DOTA) 23.

xii) Synthesis of DOTAMGly²⁸

DOTAM-Gly was synthesized using previously published methods, and was used to chelate Yb to create Yb-DOTAM-Gly 24 following the same procedure described above.
3. Results and Discussions

Scheme 4.1 demonstrates the synthesis strategies that were employed to create a DOTA-loaded resin for SPPS. Compound 14 was prepared by coupling 8 and Fmoc-Cl with activated Zn dust. Consequently, 14 was treated with 75% TFA in DCM for 40 min to remove tBu esters. The deprotected product was a complex of 15 and TFA salt, as indicated by the NMR chemical shifts of the cyclen ring that were shifted to 3.05 - 3.62 ppm, relative to the NMR chemical shifts of 2.50 - 2.79 ppm of the cyclen ring of TFA-free 14.

Compound 15 was coupled to a Wang resin 16a (sub.lev. = 1.0 mmol/g) and a glycine-preloaded Wang resin 16b (sub. lev. = 0.78 mmol/g) to create 17a and 17b, respectively. Compound 16b was prepared to demonstrate the feasibility of incorporating DOTA within a peptide sequence. Compounds 17a and 17b showed 70% loading and 83% loading, respectively, as measured quantitatively by the titration of the Fmoc group with a UV spectrometer operating at 301 nm wavelength. The lower loading of 17a relative to 17b is presumably caused by the closer proximity required by the hydrophilic 15 and the relatively hydrophobic surface of 17a, in order to couple 15 to the short linker of 17a. Adding an amino acid to the Wang resin lengthened the linker in 17b and led to an improved loading efficiency, and comparable or improved results are anticipated if the
Wang resin is pre-loaded with a longer peptide that would have even less potential for potentially adverse DOTA-resin interactions.

At the end of the coupling reaction to create 17a and 17b, an excess amount of t-BuOH was added to convert the remaining active carboxylates to t-Bu esters, and finally acetic anhydride was used to cap remaining functional active sites on the Wang resin.\textsuperscript{41,42} No active sites remained after this esterification and capping, because analysis of 21a and 21b showed no evidence of synthesized peptide without DOTA, or DOTA coupled to more than one peptide, which would be expected from any active sites that remain at this stage.

For clarity, Scheme 4.1 only shows coupling to the Wang resin at position 1 and Fmoc protection at position 4 (or 10) of the cyclen ring, and yet the Fmoc protection may also occur at position 7. This difference is inconsequential for 17a, because the resin is eventually cleaved from the DOTA to create 21a. This difference may be significant for 17b, as the two peptidyl ligands of 21b may have a 1,4 or 1,7 configuration. For comparison, studies of 1,4-DO2A vs. 1,7-DO2A have demonstrated that the 1,4 isomer makes a more stable complex with Manganese.\textsuperscript{43} Studies of RRSS and RSRS tetra(carboxyethyl) DOTA derivatives indicated different ratios of the ‘major’ square antiprismatic geometric isomer (M) vs. the ‘minor’ twisted square-antiprismatic
geometric isomer (m), although no significant differences in hydration states were observed from these particular isomers. Yet the stabilities, hydration states and water exchange rates of DOTA derivatives are known to be affected by different m/M geometric ratios. Therefore, more detailed investigations of the effects of 1,4 and 1,7 isomers on stabilities and water exchange rates of DOTA derivatives are warranted, and the SPPS methodology described herein may provide the versatility for synthesizing isomers for these investigations.

The α-bromo glycine template 2 was produced as racemates at approximately a 1 to 1 ratio, which was indicated by the NMR doublet on 5.20 and 5.46 ppm. The diastereomers of consequent steps were also determined to be the same ratio as 2. The presence of diastereomers within a peptide may raise concerns because peptide conformations critically depend on the stereochemistry of each amino acid. However, this strategy to conjugate DOTA to peptide carboxylates is intended to label a peptide with an imaging contrast agent, and is not designed so that the DOTA directly participates in the conformation or biological function of the peptide. Therefore, racemization of 2 is acceptable for molecular imaging applications that simply require DOTA-labeling of peptides. The use of brominated amino acid templates provides additional flexibility for creating peptide-DOTA imaging contrast agents, although other
brominated amino acids besides bromoglycine were not investigated in this particular study.

After removal of the Fmoc group of 17a and 17b with 20% piperidine in NMP, 17a and 17b were coupled with α-brominated CBZ-Gly-OMe\(^2\) to obtain 18a and 18b, respectively. A comparison of results from the Fmoc titration and a Kaiser test of Fmoc-deprotected 17a and 17b indicated complete removal of the Fmoc groups. Similarly, a Kaiser test of 18a and 18b indicated that loading 2 onto the resin was performed with quantitative yield.

The CBZ group is commonly used to orthogonally protect amine groups in organic syntheses. Cleavage of CBZ groups is typically performed with H\(_2\)/Pd-C or 1,4-cyclohexadiene/Pd-C in EtOH.\(^{48}\) However, these cleavage conditions are not compatible with SPPS using polymeric supports, which lead to difficult problems with separation and purification. In this study, selective CBZ cleavage conditions were tested for SPPS. BF\(_3\)·Et\(_2\)O/dimethylthioether,\(^{49-51}\) trimethylsilyl iodide\(^{52,53}\) and Et\(_2\)AlCl/thioanisole\(^{34}\) conditions were investigated during initial trials. From the results of tracing the reactions for 18a and 18b with the Kaiser test, the strong Lewis acids BF\(_3\)·Et\(_2\)O/dimethylthioether and trimethylsilyl iodide were found to rapidly cleave the Wang linker on the resin, and therefore failed to selectively cleave the CBZ group. Fortunately, the CBZ group was
found to be selectively cleaved with Et$_2$AlCl/thioanisole by reducing the temperature to -78°C and by carefully controlling the reaction time and molar ratio of Et$_2$AlCl/thioanisole to optimize reaction conditions (Figure 4.2). The reaction rates were assessed by measuring the amine contents of ~25mg of resin after the start of the reaction, by using a picric acid titration and a UV/Vis spectrometer operating at 358 nm.\textsuperscript{35} These results were compared to the measured Fmoc concentrations of 17a and 17b. To eliminate the effect of tertiary amines in the cyclen ring, the results of picric acid titration of 18a and 18b that were not subjected to Et$_2$AlCl/thioanisole was subtracted from the measurements of the cleavage reaction. Optimized conditions were determined to consist of a reaction time of 15 min at -78°C, 1 eq. of Et$_2$AlCl and 2 eq. of thioanisole relative to the CBZ concentration on the resin. Under these conditions, cleavage of the benzyl ether group of the Wang linker or incomplete CBZ cleavage limited the overall yield of 19a and 19b to 70%. For the purposes of this particular study, a 70% yield was acceptable for subsequent steps. Although cross-linking between DOTA carboxylates and amines may also account for reduced yield after CBZ cleavage, MALDI-MS analyses of 21a and 21b showed no evidence for cross-linking, which is presumably due to the sparse population of DOTA within the Wang resin.
Additional temperature- and time-dependent studies to improve this deprotection step are under investigation in our laboratory, as CBZ deprotection during SPPS has great potential for orthogonal synthetic strategies. The expected transition state is shown in Figure 4.3. Previous reports have shown that Et$_2$AlCl can bridge two carbonyl groups in a 1,3-position to form a stable 6-membered ring, which leads to bond cleavage adjacent to either carbonyl group.$^{54-56}$ However, Et$_2$AlCl would form an unstable 7-membered ring by bridging the two carbonyl groups of CBZ-Gly-OMe, and this bridge would be further destabilized by the additional planar constraints of the amide and ester groups, so that Et$_2$AlCl is more likely to form a strong interaction with just one carbonyl group. This hypothesis is confirmed by the absence of cleavage of the methyl ester group as shown in the mass spectrum of 21a and 21b, which also shows that the interaction of Et$_2$AlCl only occurs with the ester of the CBZ group.

As shown in Scheme 4.2, conventional Fmoc SPPS methods were then employed to perform step-wise coupling of amino acids to the aminoDOTA Wang resin.$^{33}$ An Asp-Glu-Val-Asp (DEVD) peptide sequence was chosen for this demonstration, because this peptide sequence is preferentially cleaved by the caspase-3 enzyme that is a focus of our molecular imaging research.$^{57}$ To enhance the coupling efficiency, two sequential applications (i.e., double coupling) of 4 equivalents of each amino acid was applied to the
SPPS after initial Fmoc deprotection. The total yield of step-wise SPPS of the four amino acids to amino-DOTA was 90% and 93% for the synthesis of 21a and 21b, respectively, as determined by weighing. Therefore the coupling efficiency for coupling the first amino acid to amino-DOTA is no lower than 93%. This high coupling efficiency indicates that the CBZ-protected amine is sufficiently exposed after deprotection, even in the presence of the bulky DOTA moiety.

The SPPS products 21a and 21b were cleaved from the resin 20a and 20b. HPLC analyses of 21a and 21b showed a single peak, indicating that the peptide was successfully synthesized without additions or deletions of amino acids. HPLC analyses showed no evidence for separating stereoisomers of 21a or 21b, or different relative ligand positions around the cyclen ring of 21b.

These SPPS products 21a and 21b were used to chelate thulium using standard conjugation methods and an Arsenazo III color test\textsuperscript{29} to create 22a and 22b. The weight of the reactants (including salt) and product indicated that the chelation was quantitative. Although the final product was not desalted to approximate isotonic conditions during subsequent analyses, the presence of salts did not affect the determination of yield. In addition, mass spectrometry did not detect the presence of 21a or 21b, further indicating that the complexation was quantitative. However, low amounts of unchelated 21a and
21b may still exist within the final product. Chelation of the lanthanide ion required lower temperatures and shorter reaction times relative to lanthanide chelations of DOTA-amide derivatives prepared in our laboratory. This facile chelation is attributed to the presence of 3 carboxylates and 1 carbonyl in 21a and 2 carboxylates and 2 carbonyls in 21b that participate in metal chelation, relative to metal chelation with amide carbonyl groups in DOTA-amide derivatives. These results are similar to detailed chelation studies of other DOTA derivatives that indicate the advantage of employing carboxylates for metal-binding,\textsuperscript{58,59} and may indicate an improved stability and reduced toxicity of 22a and 22b relative to DOTA-amide formulations. Deprotection of the methyl ester of 22a or 22b would improve chelation. This deprotection was not performed in this study, because the methyl ester group provides unique functionality for further derivatization of the peptide’s C-terminus.

To demonstrate the application of the final product for molecular imaging, 22a and 22b were detected through the effect of PARAmpagnetic Chemical Exchange Transfer (PARACEST).\textsuperscript{30,31} The PARACEST effect is created by saturating a unique NMR chemical shift corresponding to an amide hydrogen (or more generally, a hydrogen of a functional group that that exchanges with water at a rate of approximately 100 ~ 5000 sec\(^{-1}\)), and the effect is detected by observing a decrease in the water signal caused by
transferring the saturation through chemical exchange. This novel mechanism for detecting imaging contrast agents has many advantages for biomedical applications that use magnetic resonance imaging. PARACEST exploits the close proximity between the amide group and the lanthanide ion to create a very unique NMR chemical shift for the amide group of the imaging agent, which facilitates selective saturation of this chemical shift. The synthesis strategy presented in this report is specifically designed to place amide groups in close proximity to the lanthanide ion.

Selective radio frequency irradiation at -51 ppm created a 7.8% PARACEST effect from 25 mM of 22a (Figure 4.4). This PARACEST effect of 22a occurs at the same chemical shift frequency and at a similar signal strength reported for a similar compound, DOTAMGly-Tm$^{3+}$. In addition, the PARACEST effect of 22a shows good sensitivity at physiological pH and temperature, indicating that this contrast agent can be used for in vivo molecular imaging. A similar PARACEST effect of 7.3% was observed from 12 mM of 22b (Figure 4.4). The PARACEST effects from the two amide hydrogens (Hb and Hc in Figure 4.5) both occurred at -51 ppm and were not distinguishable. To investigate the basis for this similarity, molecular modeling of the DOTA core of 22b was performed with carboxylates constrained to conjugate the Tm$^{3+}$ ion. These results revealed that Hb is positioned 4.1-4.3 angstroms from Tm$^{3+}$, and Hc is positioned 4.9-5.1
angstroms from Tm$^{3+}$. Although a more detailed analysis of Tm$^{3+}$ molecular orbitals is required for an accurate analysis of NMR chemical shifts, the very similar proximities of Hb and Hc to the lanthanide ion is sufficient to justify indistinguishable PARACEST effects that are measured in relatively coarse 1 ppm increments. The PARACEST effect is proportional to the number of water-exchangeable hydrogens that have the same NMR chemical shift. 22b showed a PARACEST effect that was twice as strong as 22a (on a per molar basis), demonstrating that multiple peptidyl ligands can improve the detection sensitivity of PARACEST imaging contrast agents.

The greatest PARACEST effects of 22a and 23 were observed with a saturation power of 9.87 $\mu$T, which was the highest saturation power that was tested. However, when powers greater than 4.95 $\mu$T were used, a sloping baseline in the initially acquired portion of the CEST spectrum represented evidence for sample heating. Therefore, a 4.95 $\mu$T saturation power was used for subsequent studies, to maximize the PARACEST effect without causing sample heating. Although the equilibrium temperature can influence the PARACEST effect, only a 0.34% and 0.65% change per °C was observed for the PARACEST effect of 22a and 23, respectively (Figure 4.6).

As an example of the effect of environmental conditions, temperature can influence chemical exchange rates and therefore can modulate the PARACEST effect. However,
changes in temperature caused only a moderate change to the PARACEST effect of 22a when the temperature was changed from 25°C to 45°C. This behavior can be explained by complicated effect of temperature on water $T_1$ and $\Delta \omega$, in addition to the proton chemical exchange rate. More importantly, the effect of temperature is negligible throughout the relatively invariant range of temperatures under physiological conditions.

pH can also influence the PARACEST effect, as proton chemical exchange between water and amides is catalyzed by hydroxide ions. The amide proton showed increasingly greater PARACEST with increasing pH, reaching the greatest effect at near pH 8. The proton chemical exchange rate between an amide and water (approximately 300 Hz) is relatively slow on the MR time scale, which is characterized by the chemical shift difference between the amide and water.

Chemical exchange between amides and water is catalyzed by hydroxide ions, so that an increasing hydroxide ion concentration by an order of magnitude per pH unit accelerates this rate to improve the PARACEST effect. Conversely, the PARACEST effect of 23 decreased with increasing pH. The proton chemical exchange rate between an amine and water is approximately 3,000-5,000 Hz, which is relatively fast compared to the chemical shift difference between the amine and water (4,800 Hz at 14.1 T). An increasing hydroxide ion concentration accelerates this exchange rate, which coalesces
the chemical shifts of the amine and water so that PARACEST cannot occur.\textsuperscript{15}

Furthermore, the pKa of an amine is approximately 8.5, so that the average number of amine protons decreases as pH approaches this value. The pKa of an amide is approximately 15, so that effect of the physiological range of pH on the average number of amide protons is negligible. These pH dependencies supported the assignments of each PARACEST effect to respective functional groups, and obviated assignment to slowly exchanging water that typically exhibits a pH-invariant PARACEST effect.

The PARACEST effects of \textbf{22a} and \textbf{23} were each correlated with concentration using modified Bloch equations for two proton pools that undergo exchange (equation 4.1).\textsuperscript{62}

\[
\frac{M_s}{M_0} = \frac{1}{1 + \frac{n_{CA}[CA]T_{1sat}}{n_{H2O}[H2O]t_M}} \\
\text{ (Eq. 4.1)}
\]

\(M_s\): MR signal of water proton pool during selective saturation of the contrast agent proton pool  
\(M_0\): MR signal of water proton pool without selective saturation  
\(n_{CA}\): number of exchangeable protons for each contrast agent molecule  
\(n_{H2O}\): number of exchangeable protons for each water molecule (2)  
\([CA]\): concentration of contrast agent  
\([H2O]\): concentration of water (~55 M)  
\(T_{1sat}\): \(T_1\) relaxation time constant of the water proton pool during selective saturation of the contrast agent proton pool  
\(t_M\): average lifetime of the exchangeable proton on the contrast agent
1/T_{1sat} of 22a and 23 were each found to be linearly related to contrast agent concentration by using a T_1 inversion recovery method with selective saturation at the amide or amine chemical shifts. By substituting T_{1sat} with a linear relationship based on [CA], the modified Bloch equations can be rearranged (equation 4.2), where m and b represent the slope and intercept of the linear relationship between 1/T_1 and [CA]. This new relationship was used to determine the sensitivity of detecting 22a and 23 (Figure 4.7).

\[
\frac{1}{[CA]} = \frac{1}{(M_0/M_s - 1)} \left[ n_{CA} b n_{H_2O} \tau_M \right] - \frac{m}{b} \tag{Eq. 4.2}
\]

This double-reciprocal analysis method suffers from an emphasis on the least precise PARACEST measurements that are derived from the least concentrated samples. However, the excellent linear correlation indicated that this analysis method produced precise results.

This work is inspired by the development of relaxivity-based MRI contrast agents that undergo a permanent structural change through enzymatic catalysis that causes a change in contrast within relaxation-weighted MR images.\textsuperscript{63,64} The absolute sensitivity of relaxivity-based MR agents has been shown to be 1-2 orders of magnitude better than the sensitivities of PARACEST agents.\textsuperscript{13,39} However, the ability to selectively detect
PARACEST agents may provide additional advantages. For example, an enzymatically inert PARACEST agent with a unique saturation frequency may be directly linked to 22a to account for variances in concentration. This advantage is critical for validating caspase-3 activity detection during in vivo biomedical applications.

This synthesis methodology may also be applied to couple peptides to other metal chelators that are used for molecular imaging. For example, diethylenetriaminetetraacetic acid (DTTA) may be used in place of 8 to couple a peptide to diethylenetriaminepentaacetic acid (DTPA). To couple more than 2 peptides to a molecular imaging contrast agent, 1,4,7,10-tetraazacyclododecaneacetic acid (DO1A) may be used in place of 8 to couple 3 peptides to DOTA following the scheme to synthesize 21a, or to couple 4 peptides to DOTA following the scheme to synthesize 21b. Similarly, diethylenetriamine acetic acid (DT1A) may be used in place of 8 to couple 4 or 5 peptides to DTPA.

Larger derivatives of DOTA and DTPA, such as hexaazacyclohexadecane-N,N′,N″,N‴,N⁴,N⁵,N⁶-hexaacetic acid (HEHA) and triethylenetetraamine hexaactic acid (TTHA), provide opportunities to couple additional peptides to a single molecular imaging agent. If the chemical shifts of all amides within multiple peptide-chelator linkages have the same MR chemical shift, then exploiting this SPPS method to
link multiple peptides to a single chelator may increase the strength of the PARACEST
effect and improve the detection of these molecular imaging contrast agents.
4. Conclusions

To summarize, a new SPPS approach has been developed to couple DOTA to the C-terminus of a peptide, and to incorporate DOTA within the peptide sequence, in order to synthesize peptidyl contrast agents for molecular imaging. The selective cleavage of CBZ protecting groups in SPPS was investigated and optimized for the DOTA-loaded resin. The CBZ deprotection step and the step to load DOTA onto the resin were accomplished with acceptable yields for SPPS. Although R,S stereoisomers and 1,4 & 1,7 ligand isomers didn’t affect subsequent analyses in this report, the effect of isomeric forms on chelation stability, hydration and water exchange rates is warranted, and development of isomeric forms for studies of isomers may be facilitated by this new SPPS strategy. Peptide-DOTA products were used to chelate thulium to create PARACEST imaging contrast agents that demonstrated good detection sensitivities at physiological conditions. This synthesis strategy provides great flexibility for coupling one or more peptides to DOTA to create peptide-DOTA imaging contrast agents for many molecular imaging applications. The employment of standard SPPS techniques provides a convenient and robust method for synthesizing libraries of molecular imaging contrast agents, which may be useful for high throughput screening to identify molecular imaging contrast agents with optimal properties. Because DOTA macrocyclic rings can strongly chelate a variety
of ions, the peptide-DOTA product can be used to develop molecular imaging contrast agents for relaxivity-based MRI using Gd$^{3+}$ or Dy$^{3+}$ \cite{1,68} or for nuclear imaging using $^{64}$Cu, $^{69}$ or $^{111}$In, \cite{70} in addition to PARACEST MRI as demonstrated in this Chapter. Therefore, this synthesis strategy represents a platform technology for molecular imaging.

To summarize, DEVD-(Tm-DOTA) amide 22a shows PARACEST with good sensitivity at physiological pH and temperature, indicating that this MRI contrast agent may be used for in vivo molecular imaging. The enzyme-responsive PARACEST MRI contrast agent 22a shows good detection sensitivity at physiological pH and temperature, which establishes criteria for future in vivo applications.
5. References


Scheme 4.1. Preparation of amino-DOTA pre-loaded polymeric support. For clarity, 17a-19a and 17b-19b only show ligands coupled to the cyclen ring in a 1,4 configuration. However, a 1,7 configuration is also possible.
Scheme 4.2. Synthesis of peptidyl contrast agents. For clarity, 20a and 20b-22b only show ligands coupled to the cyclen ring in a 1,4 configuration. However, a 1,7 configuration is also possible.
Figure 4.2 The amine content following CBZ cleavage versus reaction time. For 5 eq. of Et₂AlCl/thioanisole, the reaction was too fast to control the cleavage of CBZ group and benzyl ether linker group. 1 eq. of Et₂AlCl/thioanisole showed the best CBZ cleavage conditions and a similar reaction rate was obtained with 2 eq. of Et₂AlCl. The optimized CBZ cleavage reaction was carried out for 15 min with 1 eq. of Et₂AlCl at -78°C.
Figure 4.3 Two possible transition states of Et$_2$AlCl and CBZ-aminoDOTA Wang resin. The transition state in Figure A shows the bridging of Et$_2$AlCl between two carbonyl groups. This arrangement requires the formation of an unstable 7-membered ring that also suffers from planar constraints of the amide and ester groups. Figure B represents a more stable interaction that forms a single bond between Et$_2$AlCl and one carbonyl group. This single-bond transition state is supported by mass spectral results of 21a and 21b.
Figure 4.4 The PARACEST spectra of 22a (25 mM in 5% D2O) and 22b (12 mM in 5% D2O). PARACEST spectra were acquired using a Varian Inova 600 MHz NMR spectrometer with a modified presaturation pulse sequence that included a continuous wave saturation pulse, saturation pulse power of 31 μT, and saturation delay of 4 seconds. The 1D NMR spectra of water used to construct the PARACEST spectra were acquired with saturation in 1 ppm increments from 100 ppm to -100 ppm.
Figure 4.5 The proximity of amide hydrogens to Tm$^{3+}$ in 22a and 22b. Molecular modeling results indicated that Hb is positioned 4.1-4.3 angstroms from Tm$^{3+}$, and Hc is positioned 4.9-5.1 angstroms from Tm$^{3+}$. The dotted line of the 2D schematic is 5.4 angstroms from the lanthanide ion, and is provided as a visual aid. The PARACEST effects from two different amide hydrogens Hb and Hc were not distinguishable, as only one PARACEST effect was detected at -51 ppm, which is consistent with the similar proximities of these hydrogens to the Tm$^{3+}$. 

![Diagram of molecular structures](image-url)
Figure 4.6 Dependencies of the PARACEST effects on environmental conditions. The effects of A) pH and B) temperature on the PARACEST effects of the amide group of 22a and the amine group of 23. Concentrations of 25 mM of 22a and 200 mM of 23 were used for these analyses of pH and temperature effects. The points are connected in A and least-square best-fit lines are shown in B.
Figure 4.7 The calibration of the PARACEST effect relative to concentration. Modified Bloch equations are rearranged to easily correlate concentration with the PARACEST effect (equation 2). A) Correlation for 22a. B) Correlation for 23. C) Results from the two correlations shown in A and B are used to calibrate the PARACEST effect relative to concentration for 22a (thick line) and 23 (thin line), which are nearly identical. Experimental data for 22a (open circles) and 23 (filled squares) are also shown.
Figure 4.8 $^1$H-NMR Spectrum of Cbz-Gly(OH)-OH

Figure 4.9 $^1$H-NMR Spectrum of Cbz-Gly(OMe)-OMe
Figure 4.10 $^1$H-NMR Spectrum of Cbz-Gly(Br)-OMe

Figure 4.11 $^1$H-NMR Spectrum of Fmoc-DO3A-tBu
Figure 4.12 $^1$H-NMR Spectrum of Fmoc-DO3A-tBu

Figure 4.13 $^1$H-NMR Spectrum of Fmoc-DO3A-OH
Figure 4.14 $^1$H-NMR Spectrum of Fmoc-DO3A-OH

Figure 4.15 MALDI-MASS Spectrum of DEVD-aminoDOTA 21a.
Figure 4.16 MALDI-MASS Spectrum of DEVD-aminoDOTA-Tm$^{3+}$ complex 22a.
Chapter V. Detection of Enzyme Activity Using the PARACEST Effect

Byunghee Yoo and Mark D. Pagel
A PARACEST MRI contrast Agent to Detect Enzyme Activity

1. Introduction

Proteases were first recognized for causing nonspecific tissue degradations and for nonspecific digestive activity by ubiquitously cleaving peptide bonds. Proteases are now known to also cleave specific peptide bonds that lead to specific biological processes.\(^1,2\) For example, proteases serve critical functions during many growth processes, including embryonic development, bone formation, neuronal outgrowth, wound healing and angiogenesis. Biological regulation is also mediated by proteases, including cell cycle regulation, cell apoptosis, homeostatic tissue remodeling, and immune cell migration and activation. Protease activities are critical contributors to many pathologies including cancer, arthritis, osteoporosis, neurodegenerative disorders, cardiovascular diseases, and viral infections. As a testament to the importance of this class of enzymes, proteases are estimated to be targets of 14% of current chemotherapeutic studies, even though protease genes comprise only 4.1% of the human genome.\(^3\)

In recognition of the importance of proteases in biology and biomedicine, the ‘omics’ evolution now includes the ‘proteasome’, known as the proteases and protease activities that are present during specific times or circumstances. Most studies of the proteasome have been limited to \textit{in vitro} biochemical or cell-based assays that use protein chips.\(^4\)
substrate chips,\textsuperscript{5} chemical inhibitors\textsuperscript{6} or affinity-tagged substrates.\textsuperscript{7} To overcome the inherent limitations of \textit{in vitro} studies, subsets of the proteasome have been measured within \textit{in vivo} animal models using autoquenched fluorescent inhibitors,\textsuperscript{8} FRET substrates\textsuperscript{9} and a bioluminescence reporter gene strategy.\textsuperscript{10} Yet these \textit{in vivo} optical imaging methods have limitations, particularly regarding problems with depth of penetration and coarse spatial resolutions. New \textit{in vivo} imaging methods are required to augment the armamentarium to study the proteasome.

Magnetic Resonance Imaging (MRI) compliments optical imaging by providing high-resolution images at \(~100\ \mu m\) resolution with no inherent limitations regarding tissue depth. MRI contrast agents have been developed that respond to protease activities, by incorporating iron oxide into a polymer that is subsequently degraded into monomers by the protease.\textsuperscript{11} The depolymerization causes a decrease in the nanoscale localization of iron oxide, which leads to a decrease in the agents’ $R_2$ relaxivity. This “magnetic switch” technology has several drawbacks, including potentially marginal changes in relaxivities, false negative results caused by conditions that inhibit dispersal of iron oxide such as organelle compartmentalization, and the use of large polymers during \textit{in vivo} applications. More importantly, relaxivity-based MRI contrast agents can’t be selectively detected, so that only one protease can be studied during each MRI scan.

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session, and without the benefit of including a control agent for validation.

Many recent reports have demonstrated the advantages of PARAmagnetic Chemical Exchange Saturation Transfer (PARACEST) for providing molecular-scale information using Magnetic Resonance Imaging (MRI). Some types of PARACEST agents are considered to be “smart”, because they alertly respond to changes in their molecular environments. Examples include PARACEST agents that measure tissue pH, temperature, and concentrations of glucose, lactate, arginine, or zinc. However, the modest sensitivity of PARACEST agents, often requiring a minimum concentration of 1-10 mM for adequate detection, has limited the applicability of this approach to detect endogenous molecular targets that only exist at relatively high concentrations within tissues. Furthermore, intermolecular associations may lack high specificity, which may lead to problems with interpretation of MR images.

To address these limitations, we hypothesized that enzymatic catalysis may be exploited to change the chemical structure of a high concentration of PARACEST agents and cause a detectable change in the PARACEST effect. Chemical changes caused by enzymatic catalysis are often highly specific, and rapid catalysis may allow for indirect detection of a relatively low concentration of the enzyme. Enzymatic conversion of an amide to an amine may change PARACEST by accelerating the chemical exchange rate.
between this amide/amine group and water from \( \sim 300 \text{ sec}^{-1} \) to \( \sim 3000 \text{ sec}^{-1} \).\(^{13,23}\) Also, the MR chemical shift frequency of the amide and amine will be significantly different, especially if these functional groups are proximal to a paramagnetic lanthanide ion. This chemical shift change may be especially advantageous for detection, because MR methods are very sensitive to changes in MR frequencies.

A new MRI contrast agent was summarized in Chapter II. The peptide-DOTA MR contrast agent is catalyzed by the protease caspase-3, which changes the agent’s ability to be detected via PARAmagnetic Chemical Exchange Saturation Transfer (PARACEST).\(^{24}\) This MRI contrast agent possesses an amide hydrogen that has a unique MR frequency due to the proximity of a paramagnetic lanthanide ion.\(^{25}\) Selective saturation of this unique MR frequency, followed by chemical exchange with water, causes a detectable reduction in the MR signal of the water.\(^{26,27}\) This selective saturation method provides a tremendous advantage relative to relaxivity-based MRI contrast agents, because multiple agents with unique PARACEST frequencies may be selectively detected as needed.\(^{28}\)

Many issues must be resolved before this MRI contrast agent can be applied to \textit{in vivo} studies, including optimization of detection sensitivity, timing of detection, the use of a control agent for validation of enzyme activity, and potential for detecting multiple enzymes. Furthermore, more evaluation is required to determine if this approach
constitutes a platform technology that can be applied to monitor other protease activities.

This report describes the advantages and limitations of enzyme-responsive PARACEST MRI contrast agents for studies of the proteasome.

2. Experimental Methods

i) Theory

A modified Bloch equation for two proton pools undergoing exchange was used to describe the relationship of the PARACEST effect and concentration of 22a (equation 5.1).²⁹,³⁰

\[
\frac{M_s}{M_0} = \frac{1}{1 + \frac{n_{CA}[CA]T_{1,\text{sat}}}{n_{H_2O}[H_2O]\tau_M}}
\]  
(Eq. 5.1)

\(M_s\): MR signal of water proton pool during selective saturation of the contrast agent proton pool
\(M_0\): MR signal of water proton pool without selective saturation
\(n_{CA}\): number of exchangeable protons of the contrast agent proton pool
\(n_{H_2O}\): number of exchangeable protons of the water proton pool (2)
\([CA]\): concentration of contrast agent
\([H_2O]\): concentration of water (~55 M)
\(T_{1,\text{sat}}\): \(T_1\) relaxation time constant of the water proton pool during selective saturation of the contrast agent proton pool
\(\tau_M\): average lifetime of the proton on the contrast agent
$1/T_{\text{1sat}}$ was found to be linearly related to contrast agent concentration by using a $T_1$ inversion recovery method with selective saturation at the amide or amine chemical shifts. By substituting $T_{\text{1sat}}$ with a linear relationship based on $[\text{CA}]$, the modified Bloch equation can be further simplified (equation 5.2), where $m$ and $b$ represent the slope and intercept of the linear relationship between $T_1$ and $[\text{CA}]$. This equation was exploited to determine the sensitivity of detecting contrast agent 22a.

$$\frac{1}{[\text{CA}]} = \frac{1}{\left(\frac{\text{Mo}}{\text{Ms}} - 1\right)} \left[ \frac{n_{\text{CA}}}{b n_{\text{H2O}} \tau_M} \right] - \frac{m}{b} \quad \text{(Eq. 5.2)}$$

**Figure 5.1** The correlation of concentration and PARACEST of DEVD-(Tm-DOTA) amide using modified Bloch equations. PARACEST was measured at 37 °C and pH 7.4, using a continuous wave saturation pulse applied at -51 ppm and +51 ppm at 31 µT for 4 seconds. (Concentrations: 100, 50, 25, 12.5, 6.25, 3.13 and 1.57 mM).
ii) Synthesis and characterization of the contrast agent

The contrast agents 22a and 23 were synthesized as described in Chapter III and IV. The T$_{1\text{sat}}$ relaxation time constants were measured with a 600MHz Varian Inova NMR spectrometer using an inversion recovery pulse sequence with selective saturation applied during the relaxation time and evolution time.

iii) Protease Kinetics Measurements with PARACEST MRI

PARACEST spectra of 22a were acquired by measuring the height of the water signal with the same 600 MHz NMR spectrometer, with a continuous wave saturation pulse applied for 4 seconds at each frequency in 1ppm increments. Each sample was prepared with 25 mM of 22a at 37°C and pH 7.4, and the saturation pulse was applied at 4.95 $\mu$T, unless otherwise noted for particular investigations. The PARACEST effect was optimized by arraying the saturation power from 1.24 $\mu$T to 9.87 $\mu$T. The concentration calibration used samples ranging from 1.57 mM to 100 mM. The pH was varied from values of 5.0 to 9.0 and the temperature was varied from 25.0°C to 37.0°C to investigate the effects of environmental conditions. Concentrations of 25 mM of 22a and 200 mM of 23 were used for these analyses of pH and temperature effects. The MR data were processed and plotted using VNMR (Varian, Inc., Palo Alto, CA) and Microsoft Excel.
(Microsoft Co., Richmond, WA). To account for direct saturation of water, each spectrum was fit to a single lorentzian line shape using Origin v7.5 (OriginLab Corp.). The difference between the lorentzian fit and the experimental data was used to calculate the magnitude of each PARACEST effect.

To initially test protease activity, 3.44 nM of human recombinant caspase-3 (EMD Biosciences Inc.) was added to 25 mM of 22a and 10 mM of 24 in 10 mM PIPES, 2 mM EDTA, and 0.1% CHAPS to maintain conditions for protease activity, and 5% D2O to optimize magnetic field homogeneity. PARACEST spectra were acquired before and 1 hour after adding the protease. To assess the Michaelis-Menten kinetics of the cleavage of 22a by caspase-3, 3.44 nM of the same caspase-3 enzyme was added to samples of 22a that ranged from 1.57 mM to 100 mM. The height of the water signal was then measured with selective saturation iteratively applied at ±100 ppm, ±51 ppm, and 0 ppm, and this series of saturations was repeated 45 times during a total of 75 minutes. The ratio of the PARACEST effect with saturation at -51 ppm relative to +51 ppm was used to determine the remaining concentration of 22a during the reaction. The initial reaction velocity of each reaction, vi, was determined from the first 15 min of the reaction. Each reaction was duplicated to ensure the validity of the results. A Hanes plot was used to determine the Michaelis constant, KM, and the maximum reaction velocity, Vmax.31 The
catalysis rate, $k_{\text{cat}}$, was determined from $V_{\text{max}}$ and the enzyme concentration. An identical procedure was conducted to assess the Michaelis-Menten kinetics of the cleavage of 22a with 5.84 nM of caspase-8 (EMD Biosciences Inc).

### iii) Protease Kinetics Measurements with Fluorescence Spectroscopy:

The Michaelis-Menten kinetics of the cleavage of Z-DEVD-AMC (EnzChek Caspase-3 Assay kit #1, Invitrogen Inc.) with caspase-3 was also assessed using fluorescence spectroscopy. A total of 0.172 nM of caspase-3 was added to samples of 1.57 - 100 μM Z-DEVD-AMC at 37°C and pH 7.4 for 1.5 hrs in the same buffered solution used for the PARACEST MR kinetics study. Fluorescence emission signals were measured at 441 nm following excitation at 342 nm using a fluorescence/UV plate reader (SpectraMax M2, Molecular Devices Corp.). Fluorescence was measured every 30 sec for 1 hr, and the Michaelis-Menten kinetics were analyzed as described above. Each reaction was duplicated to ensure the validity of the results. The same reaction conditions were applied to assess the cleavage of Z-DEVD-AMC with 0.292 nM of caspase-8.

### iv) Molecular Modeling

A model of 22a was constructed with CS MOPAC Pro v8.0 within Chem3D Ultra v8.0
A metal atom was inserted in the binding pocket of the model, distances between the metal and chelating amines, carboxylates, and carbonyl were constrained to 2.51 angstroms, and the resulting model was energy-minimized using the MM2 force field. A model of the structure of an Ac-DEVD-CHO inhibitor in the binding pocket of caspase-3 has been previously determined with X-ray crystallography,\textsuperscript{32} which is available as structure 1PAU within the Protein Data Bank.\textsuperscript{33} This model was modified by removing the N-and C-terminal capping groups, and connecting the $\alpha$-amino group of 23 to the C-terminus of the DEVD peptide, using InsightII (Accelrys Software Inc.). The intermolecular contacts between the protease and DOTA moiety were minimized by rotating the $\varphi$ dihedral angle of the glycyl ligand to 98.9° and the dihedral angle of the scissile bond to 166.3°. A similar molecular model of DEVD-AMC and caspase-3 was constructed, and the $\varphi$ dihedral angle of the glycyl ligand and the scissile bonds were 158.7° and 177.6°, respectively. Molecular models of DEVD-DOTA and Z-DEVD-AMC with a cis peptide bond were also constructed using the same procedure. The $\varphi$ dihedral angle of the glycyl ligand and the scissile bonds of the DEVD-DOTA model were -170.1° and -16.1°, respectively, and these dihedral angles required no distortions from -180° and 0° to accommodate the Z-DEVD-AMC model.
v) MEROPS Database Search

The MEROPS v7.30 database is an on-line information resource for peptidases, substrates, and inhibitors. The substrate search tool (“What peptidase can cleave this bond?”) was used to identify all peptidases that cleave a substrate with a N- or C-terminal blocking group, excluding the relatively small amide, ethyl ester, and methyl ester blocking groups. Each peptidase was then classified according to organism and chemical mechanism of catalysis, and compared to the same classifications of the contents of the MEROPS database (Appendix A2).
3. Results and Discussions

To demonstrate our approach, we have designed a “smart” PARACEST MRI contrast agent \textit{22a} that can detect an active caspase-3 enzyme. This enzyme is known as an “executioner” in the metabolic death cascade during cell apoptosis, and therefore serves as a critical early biomarker for evaluating apoptosis-promoting anti-tumor therapies.\textsuperscript{35} Among the identified substrates of caspase-3, DEVD (Asp-Glu-Val-Asp) is efficiently and selectively cleaved by caspase-3, and has been incorporated in fluorescence dyes for detecting caspase-3 activity such as DEVD-AMC.\textsuperscript{36,37} Our PARACEST MRI contrast agent uses a similar motif by replacing AMC with DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid).

The contrast agent DEVD-amino-(Tm-DOTA) \textit{22a}, amino-(Tm-DOTA) \textit{23}, and Yb-DOTAM-Gly \textit{24} were synthesized and characterized following procedures described in Chapter 3 and 4.\textsuperscript{28,36,38} Intermediate and final products were characterized using NMR spectroscopy and mass spectroscopy, and the structural purity of the final product was verified with HPLC as described in Chapter 2 and 3.\textsuperscript{36} The CEST spectrum of a mixture of 25 mM of \textit{22a} and 10 mM of \textit{24} showed PARACEST effects at -51 ppm and -16 ppm respectively, which were assigned to the amide most proximal to the lanthanide ion in
and \( \text{DOTAMGly} \) (Figure 5.2).\(^{28}\)

**Figure 5.2** The PARACEST spectra before and after the protease reaction. The PARACEST spectra of 25 mM \( \text{22a} \) and 10 mM of \( \text{24} \) before (open circles) and after the reaction (filled squares) with caspase-3. The PARACEST effect of \( \text{22a} \) at -51 ppm disappears after enzyme catalysis, while the PARACEST effect of Yb-DOTAM-Gly at -16 ppm remains unchanged. PARACEST effect of \( \text{23} \) at +8 ppm and the PARACEST effect of \( \text{24} \) at -16 ppm.
To show the practical application of 22a to detect the activity of caspase-3, the PARACEST spectrum of 22a was recorded by applying selective saturation in 1 ppm increments from +100 ppm to -100 ppm (Figure 5.2). A PARACEST effect was detected at -51ppm, which was assigned to the amide most proximal to the lanthanide ion in 22a, based on identical results obtained from a similar compound, Tm$^{3+}$-DOTAMGly.$^{15}$ After 48 nM of caspase-3 was added and the mixture was incubated at 37°C and pH 7.4 for 1 hour, the PARACEST effect at -51 ppm was dramatically decreased and an asymmetrical shape in the PARACEST spectrum was observed near water (0 ppm). The PARACEST effect of 22a at -51 ppm disappears after enzyme catalysis, while the PARACEST effect of Yb-DOTAM-Gly at -16 ppm remains unchanged. The Lorentzian deconvolution of the PARACEST spectrum after the reaction showed the PARACEST effect of 23 at +8 ppm and the PARACEST effect of 24 at -16 ppm. The magnitude of the PARACEST effect of 24 remained unchanged during the reaction. Considering that the PARACEST spectrum of 23 also shows an identical PARACEST peak at +8 ppm, this effect further confirms that caspase-3 has converted the DOTA-amide of 22a to the DOTA-amine of 23.

To further demonstrate the utility of 22a, a MR image with selective saturation at -51 ppm was acquired with 22a before and after reaction with caspase-3 (Figure 5.2). A
MR image with selective saturation at +51 ppm was also acquired as a control to account for direct saturation of water. The difference between these images showed a 14.5% decrease in water MR signal before the enzymatic reaction due to the PARACEST effect, and no significant change in water MR signal after reaction.

To determine the sensitivity of detecting \textbf{22a} under physiological conditions, the PARACEST effect of the agent was correlated with concentrations using modified Bloch equations. After validating a linear relationship between concentration and $T_1$ relaxation under selective saturation conditions, and confirming that the selective saturation pulse was sufficiently long to achieve steady-state conditions, this theory was further modified to obtain a linear relationship that correlates concentration to the PARACEST effect. These results indicate that 5.2 mM of the agent can be detected by saturating the amide MR frequency to generate a 5% change in water MR signal. Therefore, \textbf{22a} may be used to indirectly detect caspase-3 enzyme concentrations as low as 5-50 nM, although the minimum detection threshold will depend on the enzyme’s catalytic efficiency, other environmental conditions, and the performance of the MR scanner.\textsuperscript{39} The activities of 3.44 nM of caspase-3 and 5.84 nM of caspase-8 were successfully detected during protease kinetics measurements using PARACEST MRI (Figure 5.3).
The rate of disappearance of the PARACEST effect (i.e., slope of the line) during the first 15 minutes of each reaction was used to determine the initial reaction rate, $v_i$. The variability of these PARACEST measurements during the protease reactions scaled with the magnitude of the PARACEST effect, indicating that the signal-to-noise ratio of the NMR spectrometer remained constant and dominated variability due to the reaction conditions. The PARACEST and fluorescence kinetics analyses of caspase-8 were less precise than the same analyses with caspase-3, due to the relatively slow kinetic rates of caspase-8 compared to caspase-3 (Figure 5.4).

**Figure 5.3** Change in % water signal caused by the PARACEST effect of 22a during the reaction with caspase-3. Each line is labeled with the concentration of 22a. Each concentration was tested twice, and vertical lines represent the difference between the two measurements. The rate of disappearance of the PARACEST effect (i.e., slope of the line) during the first 15 minutes of each reaction was used to determine the initial reaction rate, $v_i$. 

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Figure 5.4 Hanes plots of Michaelis-Menten reaction kinetics.  A) The reaction with 22a and caspase-3. B) The reaction with Z-DEVD-AMC and caspase-3. C) The reaction with 22a and caspase-8. D) The reaction with Z-DEVD-AMC and caspase-8. The maximum reaction velocity, $V_{\text{max}}$, and the Michaelis constant, $K_M$, can be determined from the linear correlation in each plot.
The Michaelis-Menten kinetics parameters were determined from a Hanes plot (Table 5.1), which is less sensitive to measurement inaccuracies of initial reaction velocities at low substrate concentrations that are problematic for the Lineweaver-Burk plot or the Eadie-Hofstee plot (Appendix A1).40-42

A molecular model of 22a and caspase-3 was constructed by coupling DOTA via a trans peptide bond to the DEVD peptide within the caspase-3 binding pocket. This model showed that 22a could be accommodated within the binding pocket of caspase-3 without requiring changes to the conformation of the protease (Figure 5.5). Atomic displacements within the protease and peptidyl ligand were not made in order to preserve the geometry of the catalytic active site. Similarly, the optimized geometry of the DOTA chelate was not modified in order to preserve the metal-DOTA interactions. These restrictions only allowed for changes to the φ dihedral angle of the glycyl ligand and the scissile bond, which were distorted to 98.9° and 166.3°, respectively, to minimize interactions between the protease and the DOTA moiety. A similar molecular model of Z-DEVD-AMC and caspase-3 was constructed, and the φ dihedral angle of the glycyl ligand and the scissile bonds were 158.7° and 177.6°, respectively. In addition, molecular models of DEVD-DOTA and Z-DEVD-AMC were constructed starting with a cis peptide bond.
Figure 5.5 The molecular model of 22a and the active site of caspase-3. The solvent-accessible surface of the active site of caspase-3 is shown in white, the peptidyl moiety of 22a is shown in blue, the scissile bond is shown in yellow, the DOTA moiety is shown in red, and the lanthanide ion is shown in magenta. (A) The top view shows that the DOTA moiety can be accommodated without overlapping the sides of the active site of caspase-3. (B) The view along the “canyon floor” of the active site shows that the DOTA moiety extends away from the floor.
The φ dihedral angle and scissile bond of the DEVD-DOTA model were distorted to -170.1° and -16.1°, respectively, and these dihedral angles required no distortions from -180° and 0° to accommodate the Z-DEVD-AMC model. Although more sophisticated modeling could be conducted to further optimize intermolecular interactions and reduce the strain of the rotated dihedral angles, this simplistic model represents the gross morphology of the intermolecular interaction.

Although the minimum concentration of a contrast agent that can be detected by MRI depends in part on the signal-to-noise ratio of the scanner and sample, a 5% change in MR signal is considered to be adequate for most PARACEST MRI applications.43-46 A total of 5.19 mM of 22a or 4.47 mM of 23 is required to generate a 5% change in MR water signal at physiological conditions. More importantly, rapid protease activity can generate a relatively large change in the relative concentrations of substrates and products, which can be used to detect a relatively low amount of the protease. Figure 5.3 demonstrates that 3.44 nM of caspase-3 can be detected within 20 minutes, which is an adequate time frame for most in vivo MRI measurements.

The retention of substrates and products at the site of the protease is critical for maintaining this level of sensitivity during in vivo applications. In addition, caspase-3 is an intracellular enzyme, so that 22a must be delivered to the cell cytosol to effectively...
detect this enzyme target. To address both of these issues, the solid-phase peptide synthesis of DEVD-DOTA may be conveniently extended to add a cell penetrating peptide (CPP) sequence to the N-terminus of the DEVD peptidyl ligand.\textsuperscript{47, 48} Furthermore, CPPs have been shown to transport nanoscale cargos into cells, which provides the opportunity to deliver nanocarriers with multiple DEVD-DOTA moieties in order to accumulate a concentration of agent that is required for detection. To further address the delivery of sufficient concentrations into cells, the CPP-DEVD-DOTA contrast agent may be further extended to include a peptide that targets cells that are undergoing apoptosis. The analysis of detection sensitivity shown in this report establishes the concentrations that are required for these in vitro and in vivo studies with CPPs and the Annexin-V peptide sequence to address retention and intracellular delivery. The pH, temperature, $T_1$ relaxation time, chemical exchange rate, and concentration of water in the sample can affect the detection sensitivity of PARACEST MRI contrast agents. The PARACEST effect of $22a$ is strongest at pH values above 7, while the PARACEST effect of $23$ is strongest at pH values below 7, which provides the possibility of detecting protease activity with relatively good sensitivities throughout acidic or basic pH conditions. The PARACEST effect experiences only a small change over a physiologically relevant temperature range, although the effects of temperature must be
considered if accurate quantification of contrast agent concentrations is required. As indicated by equation 5.1, shorter $T_1$ relaxation times caused by the contrast agent will decrease the PARACEST effect, which may be relevant for intracellular water. Finally, the chemical exchange rate and concentration of water within cells may differ from the biochemical samples used in this study, which will also influence the PARACEST effect. Therefore, these results with biochemical solutions should be considered as minimum detection thresholds under favorable conditions.

The Michaelis constant, \( K_M \), measures the ability of a substrate to dissociate from the protease before a reaction can occur under specific conditions and assumptions\(^{49,50}\). The smaller \( K_M \) of the Z-DEVD-AMC:caspase-3 complex relative to the 11a:caspase-3 complex indicates a relatively stronger association between Z-DEVD-AMC and caspase-3, which indicates that the planar AMC moiety is easier to accommodate within the binding pocket of caspase-3 relative to the bulky DOTA moiety. Differences in hydrophobicities between the AMC and DOTA moieties, and the addition of the hydrophobic “Z” benzyloxycarbonyl group in Z-DEVD-AMC, may also account for the greater stability of Z-DEVD-AMC:caspase-3. The larger \( k_{cat} \) of 22a:caspase-3 relative to Z-DEVD-AMC:caspase-3 indicates that the cleavage of the scissile bond is much faster for 22a:caspase-3. The molecular models support these observations, because the
dihedral angle of the trans or cis scissile bond may be strained from planarity to reduce interactions between the protease and the DOTA moiety, which may facilitate bond cleavage. For comparison, the molecular models of Z-DEVD-AMC:caspase-3 was constructed with trans and cis scissile bonds that exhibited no or almost no distortion from planarity. Overall, this fast catalysis causes the overall catalytic efficiency of caspase-3 to be greater for 22a vs. Z-DEVD-AMC.

Table 5.1 Michaelis-Menten kinetics parameters. The results shown in Figure 5 were used to determine the kinetics parameters for reactions with caspase enzymes and fluorometric or PARACEST MRI contrast agents.

<table>
<thead>
<tr>
<th>Protease</th>
<th>Parameter</th>
<th>Z-DEVD-AMC</th>
<th>DEVD-(Tm-DOTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>Enzyme Concentration</td>
<td>0.172 nM</td>
<td>3.44 nM</td>
</tr>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$5.99 \times 10^{-9}$ M sec$^{-1}$</td>
<td>$5.52 \times 10^{-4}$ M sec$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_{\text{cat}}$ (k2)</td>
<td>$3.49 \times 10^{2}$ sec$^{-1}$</td>
<td>$1.61 \times 10^{5}$ sec$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$K_{\text{M}}$ (M)</td>
<td>$9.52 \times 10^{-5}$ M</td>
<td>$1.70 \times 10^{-2}$ M</td>
</tr>
<tr>
<td></td>
<td>Cat. Efficiency, kcat/KM</td>
<td>$3.66 \times 10^{6}$ M$^{-1}$ sec$^{-1}$</td>
<td>$9.42 \times 10^{6}$ M$^{-1}$ sec$^{-1}$</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Enzyme Concentration</td>
<td>0.292 nM</td>
<td>5.84 nM</td>
</tr>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$2.43 \times 10^{-10}$ M sec$^{-1}$</td>
<td>$7.24 \times 10^{-6}$ M sec$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_{\text{cat}}$ (k2)</td>
<td>$8.33 \times 10^{0}$ sec$^{-1}$</td>
<td>$1.24 \times 10^{3}$ sec$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$K_{\text{M}}$ (M)</td>
<td>$4.59 \times 10^{-5}$ M</td>
<td>$1.83 \times 10^{-1}$ M</td>
</tr>
<tr>
<td></td>
<td>Cat. Efficiency, kcat/KM</td>
<td>$1.82 \times 10^{5}$ M$^{-1}$ sec$^{-1}$</td>
<td>$6.77 \times 10^{3}$ M$^{-1}$ sec$^{-1}$</td>
</tr>
</tbody>
</table>
Similar comparisons are observed for similar reactions with caspase-8, although the overall catalytic efficiency of caspase-8 was more than an order of magnitude greater for Z-DEVD-AMC vs. 22a. The catalytic efficiency of 22a is almost 1400 times greater for caspase-3 relative to caspase-8. This efficiency of the PARACEST agent greatly exceeds the 20-fold greater catalytic efficiency of the fluorescence agent for caspase-3 relative to caspase-8, indicating that the bulky DOTA moiety can improve the catalytic efficiency of imaging contrast agents for particular proteases. Most importantly, the catalytic efficiency of 22a for caspase-3 establishes that enzyme-responsive PARACEST MRI contrast agents can show preferential detection of enzyme activity of individual members of the proteasome. However, the contrast agent is not absolutely selective for caspase-3 relative to caspase-8, so that high concentrations of active caspase-8 can cause the same catalysis rate as low concentrations of caspase-3, which may complicate interpretation of results.

Differential pharmacokinetics and biodistributions can complicate the evaluation of responsive contrast agents during in vivo molecular imaging applications.\textsuperscript{51} For example, the absence of a detectable PARACEST effect after administering 22a may be due to insufficient concentrations of this imaging agent in the tissue of interest, rather than the response to a protease. Fortunately, the selective detection of PARACEST
agents can be exploited to solve this problem. An unresponsive contrast agent with a unique PARACEST frequency can be combined with the responsive PARACEST agent, so that the unresponsive agent can be used to track pharmacokinetics and biodistribution. To demonstrate this approach, the protease-unresponsive agent 24 was included within the same reaction sample. No differences in the PARACEST effect at -16 ppm were detected before and after the reaction, which demonstrated that this unresponsive PARACEST MRI contrast agent can be used to monitor total concentration of the imaging agent during in vivo applications. The pharmacokinetics of each agent are very likely to be different, which can compromise this ratiometric analysis. To overcome this critical problem, the solid-phase peptide synthesis methodology used in this report can be used to couple a responsive DOTA agent to the C-terminus of a peptide sequence, and a solution-phase synthesis methodology can then be used to couple an unresponsive DOTA agent to the peptide N-terminus.36,52

Monitoring the disappearance of the PARACEST effect of 22a relative to the appearance of the PARACEST effect of 23 has advantages for monitoring protease activity. The PARACEST effect of 22a is more stable relative to 23 with respect to temperature and pH at physiological conditions. Detecting a PARACEST effect at -51 ppm avoids direct saturation of water relative to the PARACEST effect at +8 ppm. The amide
PARACEST spectrum shows a broader range of frequencies, so that multiple enzyme-responsive agents may be designed to study multiple proteases during the same MRI scan session. This is especially important for promiscuous proteases that have similar catalytic efficiencies for cleaving the same substrate, because the selective detection of two substrates can then be used to identify the two proteases. The inclusion of an unresponsive PARACEST MRI contrast agent to quantify total concentrations of each responsive contrast agent is critical for this type of Michaelis-Menten kinetics analysis of two or more proteases.

The MEROPS database is an on-line information resource for peptidases, substrates and inhibitors. A search of the MEROPS database identified 242 unique proteases that cleave a relatively large blocking group at the N- or C-terminus of a specific peptide sequence (Table 5.2). This list of proteases was further stratified to identify 88 human proteases, 30 non-human vertebrate proteases, 96 bacterial and parasitic proteases, 25 plant proteases, and 3 proteases of unidentifiable origin. The list of human proteases was further stratified to identify 18 cysteine proteases, 12 metalloproteases, 56 serine proteases, 2 threonine proteases.

Caspase-3 can be classified as an exoprotease (a.k.a. an exopeptidase), which cleaves an amide bond at the N- or C-terminus of a well-defined peptide sequence. A survey of the
MEROPS database revealed that 12.3% of the currently identified human proteasome consists of exoproteases that can accommodate substrates with relatively bulky N- or C-terminal groups (Table 5.2). This result is a conservative estimate, as all identified human proteases may not yet have been studied with substrates that incorporate bulky N- or C-terminal groups. This human ‘exoproteasome’ consists of a high percentage of serine proteases and a low percentage of metalloproteases and aspartic proteases, relative to the distribution of these catalytic mechanisms in the human proteasome. Cysteine proteases are equally represented in the ‘exoproteasome’ relative to the proteasome. This different distribution of the ‘exoproteasome’ and proteasome may reflect the relative abilities of protease types to accommodate a bulky peptide terminal group while still maintaining catalytic function. This conjecture is supported by visual inspection of all available three-dimensional structures of human proteases, which revealed a deep binding pocket in almost all structures of aspartic proteases, a shallow binding pocket in serine proteases, and more variable binding pocket depths in cysteine proteases and metalloproteases. More experimental studies of intermolecular interactions between specific peptidyl-DOTA contrast media and specific proteases are required to validate the general conclusions of this analysis of the MEROPS database. Yet this survey suggests that other proteases may be detected using this new type of contrast medium, in order to
study subsets of the proteasome.

**Table 5.2** Stratification of human proteases by catalytic mechanism. The proteasome represents all members of the MEROPS v7.30 database that have been located in the human genome. The ‘exoproteasome’ represents the results of analyzing this database to identify proteases that can cleave a bulky group from the N- or C-terminus of the peptide substrate.

<table>
<thead>
<tr>
<th>Catalytic Mechanism</th>
<th>‘Exoproteasome’</th>
<th>Proteasome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic Protease</td>
<td>0.0%</td>
<td>7.0%</td>
</tr>
<tr>
<td>Cysteine Protease</td>
<td>20.5%</td>
<td>22.6%</td>
</tr>
<tr>
<td>Metallo Protease</td>
<td>13.6%</td>
<td>30.6%</td>
</tr>
<tr>
<td>Serine Protease</td>
<td>63.6%</td>
<td>34.8%</td>
</tr>
<tr>
<td>Threonine Protease</td>
<td>2.3%</td>
<td>4.9%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>88</strong></td>
<td><strong>718</strong></td>
</tr>
</tbody>
</table>
4. Conclusions

The detection of catalytic activity of caspase-3, rather than the presence of caspase-3, has many important advantages for molecular imaging. A relatively low concentration of enzymes with rapid catalytic activities can quickly convert a high concentration of MRI contrast agents for detection using PARACEST MR methods. Similar to many other important enzyme biomarkers, caspase-3 is constitutively expressed as an inactive proenzyme, so that detecting enzyme activity avoids detection of the inactive form. The rapid catalysis of caspase-3 can be exploited to detect 3.44 nM of this protease within 15 minutes, so that a low concentration of this protease can be detected within a reasonable time frame for MRI studies. The contrast agent \textbf{22a} showed a strong preference for detecting caspase-3 relative to the similar protease caspase-8. Although the appearance of the PARACEST effect from the amine of \textbf{23} can be used to monitor the reaction, monitoring the disappearance of the PARACEST effect of \textbf{22a} has many practical advantages. To validate this disappearance, an unresponsive PARACEST MRI contrast agent should be included, which may also be used to quantify multiple proteases through Michaelis-Menten kinetics analysis. A survey of the MEROPS database indicated that this new type of contrast media may be applied to detect a broad distribution of the human proteasome. Finally, a variety of important enzyme biomarkers can catalyze the
conversion of amines, amides, and other functional groups that exchange protons with water. Therefore, this concept of a “smart” PARACEST MRI contrast agent that detects enzyme activity constitutes a fundamentally new type of molecular imaging agent that may have broad applicability for assessing enzyme biomarkers in many biological processes and disease pathologies.
5. References


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Chapter VI. Contrast agents for DCE-MRI

1. Angiogenesis and Microvasculature

Angiogenesis is the process by which new blood vessels grow into tissue from existing stromal vasculature and is tightly controlled through a balance between positive and negative blood vessel growth factors.\(^1,2\) Tumor vasculature consists of vessels recruited from the preexisting host network and new vessel growth in response to positive angiogenic factor up-regulation.\(^3-5\) Endothelial cells normally demonstrate an extremely slow rate of cell turnover. Increases in tumor growth rates from slow, linear growth to exponential growth have been observed in several tumors.\(^6\) It is postulated that an imbalance among the blood vessel growth factors released by tumor and tumor associated inflammatory cells leads to an up-regulation of angiogenesis allowing for rapid tumor growth and metastasis, often following a prolonged period of quiescence.\(^7\) Chemotactic factors involved in the angiogenic process may facilitate metastasis through incorporation of tumor cells into the growing endothelial network.\(^37\) Such factors, including collagenases, urokinases, and plasminogen activator, facilitate invasive behavior at the tips of growing capillaries. Without new blood vessel growth, it has been shown that,
when limited to simple diffusion of oxygen and nutrients, tumors grow to a maximum size of 1-2 mm$^6$ and additional tumor growth and metastasis are restricted.$^{37}$ In addition to supplying oxygen and nutrients and removing waste, endothelial cells are known to release paracrine factors that stimulate tumor cell growth. These include basic fibroblastic growth factor, insulin growth factor-2, platelet derived growth factor, and colony stimulating factor.

Increased microvascular density (MVD)$^8$ and increased vascular permeability$^{38,9,10}$ have been shown to correlate with malignancy and have been proposed as surrogate markers for angiogenesis. Increased vascular permeability in tumors is expected based on the presence of wide intercellular junctions, fenestrae, transendothelial channels formed by vesicles, and absent or discontinuous basement membranes.$^{11}$ Measurements of vascular permeability require techniques that are both labor intensive and difficult to analyze quantitatively, including perfusion with fluorescent-labeled tracers and Evans Blue dye.$^{12-15}$ The use of invasive histological techniques, however, is inherently limited. Serial measurements of MVD require repeated tissue biopsies, a procedure associated with considerable physical and emotional distress. Additionally, due to the small sample size obtained, compounded by the significant heterogeneity inherent to malignant tissues,
sampling errors may lead to inaccurate estimates of overall tumor microvascular parameters.\textsuperscript{16}

2. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI)

In angiogenesis, the microcirculation is an important factor that determines the disease progression. DCE-MRI provides important information about tissue perfusion, tissue vasculature volume, capillary permeability and interstitial volume.\textsuperscript{17 - 29} These quantitative estimates of microvascular parameters are important in characterizing the angiogenic activity of malignant tissue.\textsuperscript{30} These limitations led investigators to develop pharmacokinetic models for analyzing Dynamic Contrast-Enhanced (DCE) MRI data with the goal of developing a non-invasive, in \textit{vivo} technique for quantifying the intratumoral microvascular environment. DCE MRI has the benefit over traditional histological techniques of being non-invasive and allowing assessment of the entire tumor, thereby eliminating sampling error. It provides physiological as well as anatomical information by measuring only actively perfused blood vessel volume and permeability that reflect the current state of the tumor microvasculature.\textsuperscript{31,32} Perfusion and permeability are important characteristics of tumor vasculature during angiogenesis
and tumor growth.\textsuperscript{33-38} Quantification of perfusion and permeability has been applied to evaluate cancer treatments.\textsuperscript{39-42}

The arterial input function (AIF) is one of important factors to normalize the concentration of contrast agents in the tumor tissue with the concentration of the agents in the blood pool. But it is difficult to acquire the AIF because of the difficulties in imaging a small vein or artery and the relevance of hematocrit in artery versus tumor capillary.

A. Arterial Input Function (AIF)

DCE-MRI uses rapid imaging techniques to monitor changes in image intensity in the target tissue following intravenous (i.v.) injection of a contrast agent (CA) bolus. The temporal evolution of the CA concentration in the target tissue is extracted from the signal data assuming knowledge of CA relaxivity. By fitting the resulting concentration data to a suitable pharmacokinetic model on a pixel-by-pixel basis or in a region of interest, the maps of parameters relating to permeability/perfusion and distribution volume are acquired. To minimize variations among patients and different measurements caused by variable systemic blood supply, it is necessary to apply normalization. For this purpose, signal enhancement of healthy tissue may serve as a reference,\textsuperscript{43,44} but a more direct reference would be to measure concentration-time curves of the contrast agent in
the feeding vessels known as the arterial input function (AIF). The most commonly used physiological models in DCE-MRI are the ones requiring an AIF.\textsuperscript{45-47}

The AIF has previously been determined in humans and large animals by sampling arterial blood at multiple time points following tracer administration.\textsuperscript{48,49} However, multiple sampling of the arterial blood pool simply cannot be implemented in small animals because of the limited blood volume and short circulation times. Blood sampling rates of once every 2 sec or less are required in order to fully characterize the AIF in the mouse. It is very difficult if not impossible to withdraw blood from an arterial cannula at this rate. Furthermore, the blood volume reduction due to sampling would significantly perturb the hemodynamics of the host. MRI methods for determining the AIF have also been demonstrated in humans. These techniques extract the AIF from signal intensity changes observed in the blood of the left ventricle (LV)\textsuperscript{50,51} or in large arteries\textsuperscript{52} following injection of a CA bolus. Although several DCE-MRI studies of tumor perfusion in mice have been reported,\textsuperscript{53-55} relatively few measurements of the AIF in small animal models have been made to date. This is due in large part to the difficulties involved in making such measurements.

To determine the AIF in small animals using MR methods, a $^2$H$_2$O AIF has been measured in rats by shunting blood from a carotid artery through an RF transceiver coil
and then back into the animal. This approach yielded precise representation of the AIF, but is obviously invasive and it would be very difficult to implement in mice due to the small vessel size.\textsuperscript{56} The AIF in mice was successfully measured by wrapping a transceiver coil around the tail.\textsuperscript{57} However, this technique provides relatively low temporal resolution (75 sec) and works for blood flow rates below 0.5 cm/s, and as such is only suitable for characterizing macromolecular CAs in venous blood. In other cases, a transverse slice sectioning through the abdominal aorta and vena cava in mice has been used for quantification of vascular input function of gadopentetate, but the low sampling rate limits its utility to fully characterize the AIF.\textsuperscript{58} The AIF of $^2$H$_2$O has been measured in mice using MR spectroscopy that monitored signals from both blood and myocardium,\textsuperscript{59} but the unlocalized spectroscopic method was not able to separate contributions from the arterial blood.

\textbf{B. Contrast agents}

Blood pool agents (BPAs) are contrast agents that remain intravascular with a long blood half-life. The BPAs hence do not leak from the vessels into normal tissue in concentrations that affect MRI measurements and within the time frame of DCE-MRI experiments. To the extent that tumor vessels are leaky to the BPA, this theoretically
ensures a high diffusion gradient into poorly perfused tumor parts. Studies in experimental tumors using BPAs for DCE-MRI have shown the possibility of quantifying tumor vessel leakage of contrast agent and correlating this to histopathologically assessed vascularity and tumor grade and to DCE-MRI parameters with conventional gadolinium-based contrast agents.\textsuperscript{60 - 63} Table 6.1 lists some of the numerous contrast agents investigated for use in the quantitative characterization of tumor microvasculature using DCE-MRI.\textsuperscript{64 - 69}

<table>
<thead>
<tr>
<th>Contrast Agent</th>
<th>Characteristics</th>
<th>Mol. Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omniscan</td>
<td>Small molecular weight contrast agent</td>
<td>574 Da</td>
</tr>
<tr>
<td>Magnevist</td>
<td>Small molecular weight contrast agent</td>
<td>938 Da</td>
</tr>
<tr>
<td>MS-325</td>
<td>Gd\textsuperscript{3+} chelated contrast agent : reversibly binding to serum albumin</td>
<td>Unbound: 957 Da</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bound: ~70 KDa</td>
</tr>
<tr>
<td>P792</td>
<td>Rapid clearance</td>
<td>6.47 kDa</td>
</tr>
<tr>
<td>Gadomer-17</td>
<td>Dendrimeric</td>
<td>17 kDa</td>
</tr>
<tr>
<td>Polylsine-Gd-DTPA</td>
<td>Macromolecular</td>
<td>50 kDa</td>
</tr>
<tr>
<td>Albumin-(Gd-DTPA)\textsubscript{n}</td>
<td>Macromolecular (n≤35)</td>
<td>92 kDa</td>
</tr>
<tr>
<td>PG-Gd-DTPA</td>
<td>Macromolecular</td>
<td>228 kDa</td>
</tr>
<tr>
<td>SHU555C</td>
<td>Macromolecular</td>
<td>3–4 nm (~25nm)</td>
</tr>
<tr>
<td>Clariscan</td>
<td>Macromolecular</td>
<td>700 kDa(&lt;16nm)</td>
</tr>
</tbody>
</table>
a. Small molecular contrast agent (SMCA)

Small molecular weight gadolinium chelates are available for humans with approval and have been widely applied to estimate permeability and perfusion.\textsuperscript{70-73} Currently the small molecular contrast agents (SMCA) are the only FDA-approved contrast agents for DCE MRI, such as Gd-DTPA (Magnevist\textsuperscript{TM} and Omniscan\textsuperscript{TM}). The first-pass extraction ratios for Gd-DTPA range from 0.1-0.4\textsuperscript{74} to 1.0\textsuperscript{75} in the mammary adenocarcinoma model. With such high first-pass extravasation, these agents have demonstrated limited use in estimating microvascular volume,\textsuperscript{76} because the microvessels of malignant tumors are hyper-permeable to macromolecules with molecular weights from 15 - 30 kDa.\textsuperscript{10}

b. Intermediate molecular contrast agent (IMCA)

The molecular weights of IMCA are between 5 and 50 kDa. IMCA can be eliminated from the body by renal clearance and are also termed as “rapid clearance blood pool agents“.\textsuperscript{28,76,90} IMCA acts as blood pool agent during the first pass but its diffusion through the normal vascular endothelial membrane is limited by their size. Two different IMCAs have been investigated.\textsuperscript{66,77}

Gadomer-17 is a dendrimeric Gd chelate containing 24 Gd ions\textsuperscript{68,69,78-80} with the molecular weight of \textasciitilde17 kDa. Gadomer-17 is exclusively eliminated by the renal
filtration within 24 hours from v.i. administration. Gadomer-17 provides a higher R1 relaxivity than that of Gd-DTPA.

Gadomer-17 was investigated for the differentiation between benign and malignant breast tumors. Gadomer-17 was able to distinguish low-grade from high-grade cancers based on fractional vascular volume ($v_b$) and extravascular and whole tumor kinetics. On the contrary, it was unable to differentiate benign tumors from low-grade tumors, because of overlapping in permeability range. The mean values for transendothelial permeability and fractional plasma volume estimated from Gadomer-17-enhanced MRI data were significantly higher in malignant than in benign tumors. Gadomer-17 was applied to evaluate the therapeutic effects and showed a statistically significant difference in permeability and fractional plasma volume between pre- and post-treatments in a human breast cancer model and a mammary adenocarcinoma model.

P792 is an IMCA with a molecular weight of 6.47 kDa. It contains one Gd ion in a four armed gadolinium chelate. P792 has ~20 min of plasma half-life in rats and a R1 relaxivity of 39 mM$^{-1}$ s$^{-1}$ at 20 MHz and 37 °C. By the use of P792 and Gd-DTPA in a rodent prostate tumor model, it was expected to be possible to differentiate metastatic from nonmetastatic tumors based on contrast agent washout rates in DCE-MRI data. With the human prostate tumors model, P792 showed significant reductions in tumor
vascular permeability, tumor blood volume and tumor blood flow.\textsuperscript{86} On the contrary, with a rodent mammary tumor, P792 showed limitation in the differentiation of benign from malignant tumors based on the permeability or tumor blood volume.\textsuperscript{111}

c. Large molecular contrast agent (LMCA)

Generally, the molecular weights of LMCA are larger than the serum proteins (larger than 70 kDa or 7 nm in diameter), and LMCAs are largely circulating within the intravascular space of normal tissues with first-pass extraction fractions (<<1\%).\textsuperscript{87,88} The optimal size for LMCA suited for the assessment of tumor microvasculature, however, has yet to be determined. The LMCA should be sufficiently large not to leak across the normal vascular endothelium and small enough or degradable to be eliminated through glomerular filtration. An exclusive vascular distribution has been shown for LMCAs with sizes between 70 and 150 kDa, whereas free glomerular filtration by the kidneys was only possible below 120 kDa.\textsuperscript{89} The compounds with a molecular weight of more than 69 kDa, the weight of human albumin, have been classified as “slow-clearance blood pool contrast agents”.\textsuperscript{90} However, these agents do extravasate and accumulate interstitially within tissues with damaged or otherwise hyperpermeable microvessels.\textsuperscript{91-93}
Tumor microvessels have been long recognized to be hyperpermeable to macromolecular solutes as shown for many tumor types and by a variety of invasive techniques. These kinds of LMCAs are polylysine polymers, polysaccharides, polyglycol polyethyleneimine as well as paramagnetic dextran. LMCAs have been variably defined as paramagnetic or superparamagnetic contrast agents with molecular weights higher than 10 kDa or 30 kDa. IMCAs (Mw: 10 ~ 50 kDa) have been included by some authors in the class of LMCA. Ultrasmall superparamagnetic iron oxide (USPIO) particles have also been investigated as LMCA, although their size is considerably larger than human albumin (molecular weight 69kDa) or albumin-(Gd-DTPA). Recently, contrast agents with low molecular weights but high binding affinity to plasma proteins have been evaluated for potential to characterize tumor microvessels.

i) Albumin-(Gd-DTPA)

Albumin-(Gd-DTPA) is a prototype of a water-soluble LMCA having a molecular weight of 92 kDa (n = 30-35). It has a distribution volume of 0.05 liter/kg and a plasma half-life of three hours in rats, which produces nearly constant enhancement of normal, non-leaky tissues for 30 minutes or longer after injection. The T₁ relaxivity (R1) of albumin-(Gd-DTPA) is higher compared to low molecular gadolinium chelates. The $R_1$ of albumin-(Gd-DTPA) is 14.8 mM⁻¹s⁻¹ relative to Gd-DTPA (4.9 mM⁻¹s⁻¹).
The albumin-(Gd-DTPA)ₙ was applied to quantitatively define the characteristics of tumor microvessels, including transendothelial permeability and fractional plasma volume has been shown in several experimental cancer models, including breast, prostate and ovarian adenocarcinomas.⁠¹⁰⁷⁻¹¹⁴

Albumin-(Gd-DTPA)ₙ-enhanced MRI has also the potential to monitor the effect of antiangiogenesis therapy exemplified by anti-VEGF (vascular endothelial growth factor) antibody.¹¹⁵⁻¹¹⁷ Albumin-(Gd-DTPA)ₙ has however potent problems such as potential immunogenic properties and its prolonged partial retention in the body, i.e., 17% at two weeks.¹¹⁸,¹¹⁹

ii)  *Gadolinium chelates with reversible binding to plasma proteins*  
Small molecular gadolinium chelates with high binding affinity for plasma proteins represent a new class of MR contrast agents. The contrast agent becomes associated with a macromolecular carrier, serum albumin. The bound form assumes the macromolecular attributes of albumin and remains intravascular, while the non-bound minor component is free in the plasma and extravasates. These molecules have been recently extensively studied, and their blood persistence has been demonstrated in experimental models.¹¹⁴,¹²⁰⁻
**B22956/1** is a protein-binding contrast agent with a low molecular weight (1.06kDa).\textsuperscript{125} It has a high affinity for serum proteins. The relaxivity of B22956/1 is 27 mM\textsuperscript{-1} s\textsuperscript{-1} at 20 MHz in human serum, which is almost six-fold higher than that of conventional extravascular contrast agents. This agent can be used for monitoring the effects of an antiangiogenesis therapy with anti-VEGF monoclonal antibody.\textsuperscript{126}

**MS-325** is another protein-bound small molecular Gd chelate (MW 957 Da) with a relaxivity of 35 mM\textsuperscript{-1}s\textsuperscript{-1}. After v.i. administration, MS-325 binds reversibly to plasma albumin to form a MS-325-albumin complex with a molecular weight of ~70 kDa. This complex acts as a blood pool agent with 2-3 hrs of a plasma half-life in humans.\textsuperscript{127} The bound form is in equilibrium with a small portion of free from, which is eliminated by glomerular filtration. The binding affinity of MS-325 for albumin varies with species\textsuperscript{114,121,124} and the prolonged half-life of MS-325 provides excellent properties in a MR angiography.\textsuperscript{128}

**MP-2269** is prominent protein-binding small molecular Gd chelate of potential clinical significance. MP-2269 is a monomeric Gd-DTPA–derived blood pool agent which binds non-covalently and reversibly (80\%) to human serum albumin.\textsuperscript{120,129}
iii) *Macromolecular iron oxide compounds*

Super paramagnetic iron oxides (SPIO’s) have shown their potential for use in quantitative characterization of tumor microvasculature. SPIO shows a substantial dose-dependant increase in $T_2^*$ dephasing rate that may interfere the contrast enhancement due to $T_1$ shortening and the geometry of the microvasculature impacts the $T_2^*$ effect by increasing the rate of $T_2^*$ dephasing with increasing tracer compartmentalization. Intrinsic $T_2^*$ shortening can be partially compensated by using short echo times and lower magnetic field strengths. Compartmentalization cannot be eliminated and furthermore the effects on generated pharmacokinetic parameters are poorly understood.

SPIO’s (> 100 nm diameters) are currently applied for imaging the reticuloendothelial system, such as the liver and spleen. SPIO’s are cleared too rapidly from the blood stream (plasma half-life of 8 minutes) to evaluate the characterization of tumor microvessels. On the other hand, the ultrasmall superparamagnetic iron oxide particles (USPIO, < 30 nm diameters) are initially developed for MR lymphography and have an intravascular half-life of approximately 1-3 hours, which allow maintenance of a high blood concentration and a large enhancement in MR contrast.
**NC100150** (Clariscan) consists of USPIO particles composed of single crystals (4-7 nm diameter core), stabilized with a carbohydrate polyethylene glycol coat. The final diameter of the USPIO particles is approximately 20 nm. The R1 relaxivity of this agent is 20 mM$^{-1}$s$^{-1}$, and the R2 is 35 mM$^{-1}$s$^{-1}$ at 37 °C and 0.5 T. The mean plasma half-life in rats is about 3.3 hours. The USPIO (Clariscan) can differentiate and grade the human benign and malignant breast tumors on the basis of microvascular characteristics.$^{134,137}$

**SHU555C** is another USPIO with a mean core size of 3-4 nm and a hydrodynamic size of < 25 nm. The R1 of the agent is 22 mM$^{-1}$s$^{-1}$ of Fe, and the R2 is 65 mM$^{-1}$s$^{-1}$ of Fe (at 20 MHz and 37 °C). The mean plasma half-life in rats is about 1 hour. The potential of dynamic MRI enhanced with USPIO (SHU555C) to monitor the therapeutic effects on microvascular characteristics has also recently been demonstrated using human breast cancer xenografts.$^{116}$
3. Summary

DCE-MRI has been improved for characterizing tumor angiogenesis, based on the ability to assay microvessel permeability and fractional plasma volume. Xenograft studies have suggested that DCE-MRI can monitor antiangiogenesis cancer therapies including direct inhibitors of VEGF, antagonists of VEGF receptors, and inhibitors of matrix metalloproteases. The immediate challenge is to design a contrast agent with a sufficiently high molecular weight and size that is tolerated in humans, interacts minimally with tissue, and is stable in vivo and completely cleared from the body. SMCA and IMCA with reversible binding to plasma proteins have the advantage of providing images with a good signal to noise ratio in a relatively short imaging time. These contrast agents can be successfully applied in DCE-MRI monitoring the effects of antiangiogenic therapies. LMCA, currently in various stages of development, offer considerable promise for quantitative imaging evaluation of the tumor microvessels and antiangiogenesis therapies. The anticipated availability of a LMCA should allow the future application of DCE-MRI monitoring techniques from experimental to wide scale clinical implementation.

Currently, however, $T_1$ relaxivity based contrast agents have limitations in the acquisition of arterial input function (AIF), the administration sequence of contrast agents and long
scan time. In real experimental situations, it’s difficult or impossible to acquire AIF resulting in inaccurate concentration profile of contrast agents in target tissue. Despite many challenges, it’s still one important factor to be resolved. When two contrast agents are used for DCE-MRI, LMCA and SMCA should be administered separately and only one contrast agent can be monitored at one scan session.

These limitations in previous DCE-MRI methods could be overcome by the use of PARACEST contrast agents. By selective irradiation of MR frequencies for each contrast agent, two or more contrast agents can be monitored at the same time. It means two or more contrast agents can be administered in the body at the same time and monitored during one scan session. Also, based on PARACEST contrast agents, the concentration of each contrast agent can be normalized by calibration curves of each contrast agent after modifying the effect of physiological environments. The AIF can be removed from the complicated equations to extract the parameters in DCE-MRI experiments. Chapter VII provides an experimental evaluation of PARACEST DCE-MRI.
4. References


35. Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J. Natl Cancer. Inst.* 1990, 82, 4-6.


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111. Turetschek K, Floyd E, Shames DM, et al. Assessment of a rapid clearance blood pool MR contrast medium (P792) for assays of microvascular characteristics in


Chapter VII. PARACEST DCE-MRI
1. Introduction

The standard DCE-MRI protocol is limited to the use of just one MRI agent per scan session as shown in Chapter VI. We hypothesize that multiple MRI agents can be selectively detected using the PARACEST effect within *in vivo* animal tumor models during a modified DCE-MRI protocol, which can more accurately determine vascular permeability, extravascular plasma volumes, and vascular plasma volumes. To test this hypothesis results from a new protocol employing the PARACEST effect will be compared to results from a standard T₁ DCE-MRI protocol to assess the improvement in vascular permeability measurements. Based on published results that detail the methods for detecting PARACEST-based MRI agents, modification of the DCE-MRI protocol for detection of PARACEST agents is anticipated to be straightforward. Vascular permeability using a standard MRI agent is traditionally represented by $K^{\text{TRANS}}$, the permeability-surface area volume transfer constant between vascular plasma and extracellular space. $K^{\text{TRANS}}$ can be determined from the change in MRI agent concentration when permeability is low relative to blood flow. This measurement is dependent on MRI acquisition parameters and the blood composition, which compromises measurement accuracies and complicates comparisons between measurements. This research will introduce $\Delta K^{\text{TRANS}}$, the *relative* vascular permeability-
surface area transfer constant. \( \Delta K^{\text{TRANS}} \) is determined from concentration changes of two MRI agents. One MRI agent effectively serves as an internal reference for the other MRI agent within this new algorithm, so that this relative measurement is independent of the MRI acquisition parameters and the blood composition, which will provide superior measurement accuracies and comparisons relative to current methods.

2. Experimental Methods

A. Synthesis of Molecular Imaging Probes

i) DOTAMGly ethyl ester (DOTAMGly-OEt)

The DOTAMGly ligand\(^{1-3}\) was prepared by following Scheme 7.1. Firstly, N-(2-Bromoethanoyl) ethyl glycinate was synthesized from bromoethanoyl bromide (10.5ml, 0.12mol) and glycine ethyl ester (14g, 0.1mol). N-(2-Bromoethanoyl) ethyl glycinate (9.19g, 41mmol) was coupled to cyclen (1,3,5,7-tetraazacyclododecane, 1.72g, 10mmol) to yield DOTAMGly-OEt, which was synthesized by exhaustive alkylation of cyclen with N-(2-Bromoethanoyl) ethyl glycinate in the presence of K\(_2\)CO\(_3\) (11.06g, 80mmol) as base. The reaction was carried out in acetonitrile by heating at 70°C for 6 hrs under N\(_2\) purging. The solution was cooled to room temperature, and the undissolved materials
were removed by filtration. The product was obtained by evaporating the solvent (7.40g, quantitative yield).

**ii) DOTAMGly**

To convert the ethyl ester of DOTAMGly-OEt (7.45g, 10mmol) to a carboxylic acid, the hydrolysis reaction was carried out in ethanol/water (1:1) at 60°C by controlling pH with 1N-NaOH solution (pH~11). The reaction was traced with TLC and it was completed after 1h. The reaction mixture was cooled and acidified to pH 3 with 1N-HCl. The solution was lyophilized to produce solids. To purify DOTAMGly, the solids were dissolved in small amounts of water and separated from the solution by liquid chromatography (solid-phase: Amberlite® XAD-1600; eluent: water, 5.04g, yield: 80%).

**iii) Lanthanide complexation with ligand (Ln-DOTAMGly)**

DOTAMGly (633 mg, 1mmol) was dissolved in water (5 mL) at pH 6.5 and 40 °C, and TmCl₃ (270mg, 1mmol) or YbCl₃ (273 mg, 1 mmol) in water (3 mL) was added drop by drop for 1 hr and adjusted to pH 7.5 with 0.1-N NaOH solution (Figure 7.1). The solution was stirred for 12 hrs at 60 °C and adjusted to pH 7.5 whenever the pH dropped below 5. The reaction mixture was cooled to room temperature. The pH was adjusted to
9 and the residual lanthanide-hydroxide white precipitate was removed by filtration. The complete complexation was evaluated with an Arsenazo III color test, which showed negative results for lanthanide free condition. The solution was freeze dried, yielding Tm-DOTAMGly (750mg, yield: 94%) or Yb-DOTAMGly (755mg, yield: 94.5%).

iv) Albumin-Yb-DOTAMGly

Yb-DOTAMGly (400mg, 0.5mmol), was activated by water soluble carbodiimide, 0.15g of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide • hydrochloride) and 30mg of NHS (N-hydroxy succinimide) in 5ml of MES (2-Morpholinoethanesulfonic acid) buffer (pH=5) in an ice bath (4°C) for 1 hour. 0.34g of human serum albumin (HSA) was dissolved in MES buffer and kept in an ice bath with stirring. The activated Yb-DOTAMGly-EDC adduct was added in a dropwise manner for 30 minutes into HSA solution. The reaction was completed after 6 hours and the solution was dialyzed to purify the product. The dialysis was carried out by use of cellulose membrane tubing with a cut-off range MW 5000 for 1 day. The dialyzed solution was freeze dried to obtain albumin-Yb-DOTAMGly as white fluffy solid (0.63g, yield: 79%). The coupling efficiency (the ratio of HSA/Yb-DOTAMGly was measured by MALDI-Mass spectrometer and compared with HSA as control.
v) *Albumin-Gd-DTPA*

Gd-DTPA was acquired by lyophilization of commercially available 1M Gd-DTPA solution (Magnevist™). The obtained white solid was dissolved in 5ml of MES buffer with 0.15g of EDC and 30mg of NHS in an ice bath (4°C). After 1 hour of activation, the Gd-DTPA-EDC adduct was added in a dropwise manner for 30 minutes into the human serum albumin (0.34g) solution. The reaction was completed after 6 hours and the solution was dialyzed to purify the product. The dialysis was carried out by use of cellulose membrane tubing with a cut-off range MW 5000 for 1 day. The dialyzed solution was freeze dried to obtain albumin-Gd-DTPA as white fluffy solid (0.55g, yield: 68%). The coupling efficiency (the ratio of HSA/Gd-DTPA) was measured by MALDI-Mass spectrometer and compared with HSA as control.
B. Cell culture and Flank Tumor Model

i) Cell culture of MDA-MB0468 and MCF7

Tumor mouse models were prepared using MCF7 and MDA-MB-468 human mammary carcinoma cells. MCF7 was cultured with RPMI1640 medium with 5% FBS and 1% Pen/Strep, and MDA-MB-468 was cultured with Lebovich L-15 medium with 10% FBS. Both cell cultures were incubated at 37 °C under a 5% CO2 environment. The medium was changed 3 times per week and trypsinized for sub-culturing once per week.

ii) Tumor Implantation

Flank tumor models were prepared by suspending 10^6 cells of MDA-MB-468 or MCF7 cells in 0.25 mL HBSS medium and homogeneously mixed with the same volume of Matrigel™. The 0.5 mL volume was injected subcutaneously at the right lower flank of female nude mice. The syringe with the cell/Matrigel suspension was warmed to 30~35°C before injecting to ensure that the injection didn’t move in the body. Tumors were allowed to grow to a size of 0.7 x 0.7 mm. Usually the tumors grew to this size 2 weeks after the injection.

C. PARACEST DCE-MRI

i) Procedure for catheterization on Tail Vein

To perform DCE MRI, each mouse was anesthetized with isoflurane in 100% oxygen gas,
a 26g catheter will be inserted into a tail vein to administer MRI agents, the mouse and catheter were secured within a customized animal cradle, and the flank tumor was immobilized to minimize motion artifacts during the DCE MRI protocol. Core body temperature and respiration rate were monitored throughout the MRI session using physiological monitoring instrumentation (SA Instruments, Stony Brook NY), and automated temperature control was used to maintain physiological stability. Only one DCE MRI protocol, using multiple CEST-based MRI agents or just one standard MRI agent, was conducted for each mouse. After the DCE MRI session, the anesthetized mouse was euthanized via asphyxiation.

\[ ii) \textit{Measurements of PARACEST DCE-MRI} \]

The temporal profile of PARACEST-based MRI agent uptake in the flank tumor was obtained using a 2D spin echo (RARE spin echo) acquisition protocol to minimize RF inhomogeneities and magnetic field susceptibilities. An initial 90° excitation pulse was used to obtain steady state signal amplitudes within the first MRI acquisition. The frequency of the PARACEST exchange site of the MRI agent to be detected was saturated prior to the initial excitation pulse. Five pre-injection image sets were acquired to establish a baseline without the PARACEST effect, the MRI contrast agents in
heparinized saline were injected through the tail vein catheter, and image sets were acquired for 60 minutes with the saturation of PARACEST exchange site frequencies.

The concentration of the PARACEST-based MRI agent in the injection solution was validated by comparing PARACEST spectra of the injection solution to PARACEST spectra of standard solutions with concentrations calibrated via 9.4 T MR scanner (Table 7.1).

Table 7.1 The loading amounts of each contrast agents

<table>
<thead>
<tr>
<th>Contrast agent</th>
<th>Injection conditions</th>
<th>Injection solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd-DTPA</td>
<td>0.2 mmol/kg</td>
<td>100 mM in 50uL</td>
</tr>
<tr>
<td>Albumin-(Gd-DTPA)</td>
<td>0.03 mmol/kg</td>
<td>15 mM in 50uL</td>
</tr>
<tr>
<td>Tm-DOTAMGly</td>
<td>0.4 mmol/kg</td>
<td>100 mM in 100uL</td>
</tr>
<tr>
<td>Albumin-(Yb-DOTAMGly)</td>
<td>0.06 mmol/kg</td>
<td>15 mM in 100uL</td>
</tr>
</tbody>
</table>

The temporal profile for standard LMCA uptake in the flank tumor was obtained using a T₁-weighted sequence (MSME), and without frequency saturation prior to the initial excitation pulse. Other experimental conditions were identical to the protocol used for detecting PARACEST-based MRI agent uptake. This experiment was repeated using a standard SMCA (Gd-DTPA) at an injection of 0.03 mmol/kg. The concentration of each contrast agent in the injection line was validated by comparing R₁ relaxation rates of the
injection solution to \( R_1 \) relaxation rates of standard solutions with concentrations calibrated via 9.4 T MR scanner. Gd-DTPA and Albumin-(Gd-DTPA) were used for \( T_1 \) weighted DCE-MRI.

### D. Analysis of DCE-MRI data

**i) Analysis of \( T_1 \) DCE-MRI**

Most methods of analyzing dynamic contrast-enhanced \( T_1 \)-weighted data have used a compartmental analysis to obtain some combination of the two principle parameters: the transfer constant (\( K^{\text{Trans}} \)) and the extravascular extracellular space (EES) fractional volume (\( v_e \)).

The transfer constant and the EES relate to the fundamental physiology, whereas the rate constant is the ratio of the transfer constant to the EES:

\[
k_{ep} = \frac{K^{\text{Trans}}}{v_e}
\]

(Eq. 7.1)

The rate constant can be derived from the shape of the tracer concentration vs. time data, whereas the transfer constant and EES require access to absolute values of tracer concentration.

A tracer flows passively from the blood plasma in a permeable capillary into the EES, through microscopic pores or defects in the capillary walls. This has also been called the
‘‘interstitial water’’ or ‘‘interstitial space.’’ Note that ‘‘extravascular’’ is included in the term, to exclude specifically the blood plasma space (which is technically part of the whole extracellular space). The rate constant \(k_{ep}\) is formally the flux rate constant between the EES and blood plasma. Both the transfer constant and the rate constant have the same units (\(\text{min}^{-1}\)) and can easily be confused. The rate constant \(k_{ep}\) is always greater than the transfer constant \(K_{\text{Trans}}\). For a range of typical EES fractional volumes seen in tumors (\(v_e \sim 5\%–10\%\)) and multiple sclerosis (\(v_e \sim 20\%–50\%\)), \(k_{ep}\) is two to ten times higher than \(K_{\text{Trans}}\) (see equation 7.1).

Kety produced a model of flow-limited tracer uptake in tissue that has been used extensively. It was developed for the case of breathing an inert gas, which distributes into the whole tissue, including the intracellular spaces. Its first assumption is that arterial and venous blood have well-defined concentrations, supplying and draining the tissue under study. Second, because permeability is high, venous blood leaves the tissue with a tracer concentration that is at all times in equilibrium with the tissue. Thus, soon after injection of the tracer, the arterial concentration is high, the venous concentration is low, and most of the tracer is being removed from the blood as it passes through the tissue. For an extracellular tracer, the Kety model can be extended by setting the venous concentration equal to that of the EES. The effect of intravascular tracer on the MR signal is ignored.
(i.e., the vascular signal is small compared with the tissue signal). In this case the following differential equation relating tissue concentration $C_t$ to arterial plasma concentration $C_p$ is obtained:

$$\frac{dC_t}{dt} = F \rho (1 - Hct)(C_p - \frac{C_t}{v_e}) \quad \text{(Eq. 7.4)}$$

$$C(t) = \lambda k_{ep} \int_0^{t'} C_a(\tau) e^{-k(t-\tau)} d\tau \quad \text{(Eq. 7.5)}$$

$$k = EF / \lambda \quad \text{(Eq. 7.6)}$$

$$E = 1 - e^{PS/F} \quad \text{(Eq. 7.7)}$$

The transfer constant $K^{\text{Trans}}$ has several physiologic interpretations, depending on the balance between capillary permeability and blood flow in the tissue of interest. In high-permeability situations (where flux across the endothelium is flow limited), the transfer constant is equal to the blood plasma flow per unit volume of tissue:

$$K^{\text{Trans}} = F \rho (1 - Hct) \quad (PS >> F) \quad \text{(Eq. 7.2)}$$

In the case of low permeability, where tracer flux is permeability limited, the transfer constant is equal to the permeability surface area product between blood plasma and the EES, per unit volume of tissue:

$$K^{\text{Trans}} = PS \rho \quad (PS << F) \quad \text{(Eq. 7.3)}$$
Note that the other quantities in this equation ($F$, $\rho$, $Hct$, and $v_e$) are constants for the tissue (Table 7.2 and Table 7.3).

### Table 7.2. Three Standard Kinetic Parameters in DCE-MRI

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Units</th>
<th>Full Name</th>
<th>Alternatives</th>
<th>Discontinued Terms</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{\text{trans}}$</td>
<td>Transfer constant\textsuperscript{a}</td>
<td>min\textsuperscript{-1}</td>
<td>Volume transfer constant between blood plasma and EES</td>
<td>EF, FE, CL\textsubscript{d}/$V_t$\textsuperscript{b}</td>
<td>$K$, $k^{PS\rho}$</td>
</tr>
<tr>
<td>$k_{cp}$</td>
<td>Rate constant</td>
<td>min\textsuperscript{-1}</td>
<td>Rate Constant between EES and blood plasma</td>
<td>$k_2$, 1/$\tau$\textsuperscript{c}</td>
<td></td>
</tr>
<tr>
<td>$v_e$</td>
<td>EES\textsuperscript{d}</td>
<td>None</td>
<td>Volume of extravascular extracellular space per unit volume of tissue\textsuperscript{e}</td>
<td>Interstitial space, leakage space</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}If permeability is high (PS>>F, i.e., Kety model), this is the blood plasma flow per unit volume of tissue ($K_{\text{trans}} \sim F\rho(1/Hct)$). Of permeability is low (PS<<F), this is the permeability surface area product per unit volume of tissue, for transendothelial transport between plasma and EES ($K_{\text{trans}} \sim PS\rho$).

\textsuperscript{b}EF is the extraction flow product; $CL_d$=clearance.

\textsuperscript{c}$k_2$, 1/$\tau$

\textsuperscript{d}EES = Extracellular extracellular space.

\textsuperscript{e}ie, the volume fraction of the EES
### Table 7.3 Working Quantities in DCE-MRI

<table>
<thead>
<tr>
<th>Quantities</th>
<th>Definition</th>
<th>unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_a$ or $C_a(t)$</td>
<td>Tracer concentration in arterial whole blood $^a$</td>
<td>mM $^b$</td>
</tr>
<tr>
<td>$C_p$</td>
<td>Tracer concentration in arterial blood plasma $^a$</td>
<td>mM</td>
</tr>
<tr>
<td>$C_t$ or $C(T)$</td>
<td>Tracer concentration in tissue</td>
<td>mM</td>
</tr>
<tr>
<td>$E$</td>
<td>Initial extraction ratio $^c$</td>
<td>None</td>
</tr>
<tr>
<td>$Hct$</td>
<td>Hematocrit</td>
<td>None</td>
</tr>
<tr>
<td>$F$</td>
<td>Perfusion (or flow) of whole blood per unit mass of tissue</td>
<td>ml g$^{-1}$ min$^{-1}$</td>
</tr>
<tr>
<td>$P$</td>
<td>Total permeability of capillary wall</td>
<td>cm min$^{-1}$</td>
</tr>
<tr>
<td>$PS$</td>
<td>Permeability surface area product per unit mass of tissue</td>
<td>ml min$^{-1}$ g$^{-1}$</td>
</tr>
<tr>
<td>$S$</td>
<td>Surface area per unit mass of tissue</td>
<td>cm$^2$ g$^{-1}$</td>
</tr>
<tr>
<td>$V_b$</td>
<td>Total whole blood volume $^f$</td>
<td>mL</td>
</tr>
<tr>
<td>$V_e$</td>
<td>Total EES volume $^f$</td>
<td>mL</td>
</tr>
<tr>
<td>$V_p$</td>
<td>Total blood plasma volume $^f$</td>
<td>mL</td>
</tr>
<tr>
<td>$V_t$</td>
<td>Total tissue volume $^f$</td>
<td>mL</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Tissue blood partition coefficient</td>
<td>mL g$^{-1}$</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Density of tissue</td>
<td>g mL$^{-1}$</td>
</tr>
</tbody>
</table>

$^aC_a = (1/Hct)C_p$

$^b1$ mM = 1 mmole/liter

$^c$ EES = extravascular extracellular space

$^d1$ mL = 1 cm$^3$

$^e$ Extraction; extraction fraction is an alternative name

$^f$ NB $V_b$=$v_bV_t$; $V_e$=$v_eV_t$; $V_p$=$v_pV_t$=$(1/Hct)V_b$

$^g\lambda = fPF/(1-Hct)$; $fPF$ = fraction volume of extracellular extravascular space ($V_e$)
ii) Analysis of PARACEST DCE-MRI

In the two compartment model, a graphical analysis method has been developed for tracers with reversible uptake.\textsuperscript{12} For such tracers, after a suitable time $t^*$ by which all reversible compartments have equilibrated with the arterial plasma, the following integral equation can be used to relate the time courses of uptake of tracer in tissue and plasma.\textsuperscript{13,14}

\begin{align*}
C_t(t) &= K_{\text{Trans}} \int_0^t e^{-k_{\text{pe}}(t-\tau)} C_p(\tau) d\tau + v_e C_p(t) \quad \text{(Eq. 7.8)} \\

\end{align*}

$v_e$: combined fractional volume of plasma and rapidly equilibrating non-plasma spaces

$K_{\text{Trans}}$: transvascular outward directional transfer constant

$k_{\text{pe}}$: transvascular inward directional transfer constant

$C_p(t)$: concentration of contrast agent in plasma as a function of time

$C_t(t)$: concentration of contrast agent in tumor as a function of time

The tumor response to a short arterial pulse of concentration $= 1/(\text{pulse duration})$, i.e., a delta function, is set as

\begin{align*}
h(t) &= K_{\text{Trans}} e^{-k_{\text{pe}}t} \quad \text{(Eq. 7.9)} \\

\tau &= \frac{\int_0^\infty th(t) dt}{\int_0^\infty h(t) dt} = \frac{K_{\text{Trans}} \int_0^\infty te^{-k_{\text{pe}}t} dt}{K_{\text{Trans}} \int_0^\infty e^{-k_{\text{pe}}t} dt} = 1/k_{\text{pe}} \quad \text{(Eq. 7.10)}
\end{align*}
$C_i(t) = K_{\text{Trans}} e^{-k_{ep}(t-1/k_{ep})} \int_0^t C_p(\tau)d\tau + \nu_v C_p(t)$  \hspace{1cm} (Eq.7.11)

divide by $C_p(t)$,

$$\frac{C_i(t)}{C_p(t)} = K_{\text{Trans}} e^{1-k_{ep}} \left( \int_0^t \frac{C_p(\tau)d\tau}{C_p(t)} \right) + \nu_v \hspace{1cm} (Eq.7.12)$$

$$\frac{C_i(t)}{C_p(t)} = K_{\text{Trans}} \cdot C \cdot Patlak(t) + \nu_v \hspace{1cm} (Eq.7.13)$$

$C$ : constant including MRI acquisition parameters

$K_{\text{Trans}}$ determines the amplitude of the initial response (the amount of contrast agent that enters extravascular extracellular space (EES) and $k_{ep}$ determines the washout rate from the EES back into the blood plasma.

In the case of measuring concentrations of two contrast agents with different sizes, the following ratio can be determined.

Measuring concentrations of two CEST agents with different sizes provides a ratio:

$$\frac{C_{1,p}(t)}{C_{2,p}(t)} / C_{2,p}(t) = \frac{K_{1,\text{Trans}} \cdot C_1 \cdot Patlak_1(t) + \nu_{1,e}}{K_{2,\text{Trans}} \cdot C_2 \cdot Patlak_2(t) + \nu_{2,e}} \hspace{1cm} (Eq.7.14)$$

assuming:

$C_{1,p}(t) = C_{2,p}(t)$

$C_1 = C_2$

$Patlak_1(t) = Patlak_2(t)$

$K_{i,\text{Trans}} \gg \nu_{i,D} / C \cdot Patlak_i(t)$ \hspace{0.5cm} (i = 1, 2)
Therefore, $\Delta K_{\text{Trans}}$ is independent of the MRI acquisition parameters and the blood pool composition. To satisfy the assumptions, the measurement must be made within a relatively long time window after the CEST agent injection; this is also required for analysis of relaxivity-based macromolecular contrast agents due to their relatively low $\Delta[\text{CA}]_T$ over time.

and set:

$$\Delta C_i = \frac{C_{1,2}(t)}{C_{2,2}(t)}$$

then

$$\Delta[\text{CA}]_T = \Delta C_i = \frac{K_{1,\text{Trans}}}{K_{2,\text{Trans}}} = \Delta K_{\text{Trans}}$$

(Eq. 7.15)
3. Results

A. Contrast agents

The large molecular contrast agent (LMCA) and the small molecular contrast agent (SMCA) were characterized for the application to DCE-MRI. The relaxivity of each LMCA and SMCA is summarized in Table 7.4.

Table 7.4 Relaxivities of contrast agents for DCE-MRI experiments

<table>
<thead>
<tr>
<th>Lanthanide Set</th>
<th>Relaxivity (1/mM·sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd-DTPA</td>
<td>4.14</td>
</tr>
<tr>
<td>Alb-Gd-DTPA</td>
<td>221.71</td>
</tr>
<tr>
<td>Yb-DOTAM-Gly</td>
<td>0.0106</td>
</tr>
<tr>
<td>Alb-Yb-DOTAM-Gly</td>
<td>0.9844</td>
</tr>
<tr>
<td>Tm-DOTAM-Gly</td>
<td>0.0567</td>
</tr>
<tr>
<td>Alb-Tm-DOTAM-Gly</td>
<td>1.09</td>
</tr>
</tbody>
</table>

For the PARACEST DCE-MRI, Tm-DOTAMGly is used as a SMCA and calibrated within the range of 2 mM ~ 20 mM for the quantitative analysis of DCE-MRI results (Figure 7.1). To prepare a LMCA, human serum albumin (HSA) was coupled with Yb-DOTAMGly and the number of Yb-DOTAMGly coupled to each HSA was 13, which was verified by MALDI mass spectrometry by comparing HSA with the control. The
concentration versus % CEST effect of LMCA is shown in Figure 7.2.

**Figure 7.1** Calibration curve and linear fitting for Tm-DOTAMGly based on equation 5.1 and 5.2.
Figure 7.2 Calibration curve and linear fitting for Albumin-(Yb-DOTAMGly) based on equation 5.1 and 5.2.
B. Tumor model and catheterization

The cultured 1 million cells of human breast cancer in 0.25 ml HBSS were mixed the same volume of Matrigel™. The mixture was kept in 4°C in ice bath for the mixing and transfer. At this temperature, the viscosity is low, which causes small size tumor or non-tumored mice or tumors with variable locations in many cases. To overcome this problem, the injection mixture was heated to room temperature before the injection, it shows a gel-like appearance and implanted successfully on the very point injected. A total of 0.5 ml was injected subcutaneously at the right lower back of female nude mice (20 female nude mice, age 3 weeks, weight 25g). After 3 weeks of tumor growth, the mice were used for DCE-MRI.

For DCE-MRI measurements, the mice were catheterized at the tail vein (Figure 7.3 A). The catheterized mouse was transferred to a mouse cradle for MR measurements (Figure 7.3 B). The mouse was anesthetized by the use of isoflurane in a gas chamber. The tail was immersed in warm water (45°C) for 5 minutes and catheterized with heparin filled catheter at the tail vein. The injection line was filled with 100 μL of SMCA-50 μL of saline-100 μL of LMCA sequentially (Figure 7.4) and connected to the catheter. The dead volume of connector and needle was 80 μL and the first injection was 180 μL considering those dead volumes.
Figure 7.3 Catheterization in tail vein and a mouse cradle for MR scanner.
C. $T_1$ DCE-MRI

$T_1$ DCE-MRI was performed using a Bruker 9.4 T MR scanner with a 20 cm bore and a 35 mm birdcage coil. Each mouse was placed in the magnet and the breathing rate and the body temperature were monitored. During MR scanning, the mean breathing rate was kept below 50 min$^{-1}$ and the temperature was maintained as 37.0 °C using warmed air (SA Instruments, Inc.). Axial $T_1$-weighted MSME (Multi Slice Multi Echo) sequence was used with these settings: TE 1.4 ms, TR 200 ms, FOV 40 mm x 40mm, 258 x 258 matrix and effective slice thickness of 1 mm. The LMCA was monitored for 40 minutes and the SMCA was monitored for 20 minutes. The results are shown in Figure 6.6. From the
acquired T₁ DCE-MRI data sets in Figure 7.5 (A). The MR intensities were correlated to
determine R₁(t) using equation 7.16 and the results are shown in Figure 7.5 (B). The
relaxation time of tumor before injection was 2.5 sec and the relaxivities of SMCA and
LMCA are 4.14 and 221.71 respectively. The obtained concentrations of contrast agent in
the tumor were correlated with time to calculate $K^{\text{Trans}}$ values as shown in Figure 7.8 (A),
and $\Delta K^{\text{Trans}}$ is obtained as 0.031.¹⁵

\[
1 - e^{TrR_t(t)} = \frac{I_t(t)}{I_t(0)}
\]  
(Eq. 7.16)

\[
\Delta R_t = R_t(t) - R_t(0)
\]  
(Eq. 7.17)

\[
[CA] = \frac{\Delta R_t}{\text{Relaxivity}}
\]  
(Eq. 7.18)

D. PARACEST DCE-MRI
PARACEST DCE-MRI was performed using Bruker 9.4T MR scanner. The mice were
placed in the magnet and the breathing rate and the body temperature were monitored.
During MR scanning, the mean breathing rate was kept below 50 min⁻¹ and the
temperature was maintained as 37.0 °C using warmed air (SA Instruments, Inc.). An axial
T₂-weighted RARE (Rapid Acquisition with Relaxation Enhancement) sequence was
used with these settings: TE 41.9 ms, TR 5000 ms, FOV 40 mm x 40mm, 258 x 258 matrix, effective slice thickness of 1mm, saturation power 21uT. The in vivo pharmacokinetics of the LMCA and the SMCA were monitored for 60 minutes in selective saturation conditions. Before the injection of each contrast agent, off resonance images were measured at +51ppm for SMCA and +16ppm for LMCA. The obtained PARACEST DCE-MRI results are plotted as the % CEST change versus time (Figure 7.6 (A) and 7.7 (A)). The concentration of contrast agents are calculated from the calibration curves (Figure 7.6 (B) and 7.7 (B)). The obtained concentrations of contrast agent in the tumor were correlated with time to calculate $K_{\text{Trans}}$ values as shown in Figure 7.8 (B), and $\Delta K_{\text{Trans}}$ is obtained as 0.105 by the use of equation 7.15.

The $K_{\text{Trans}}$ values from T1-DCE-MRI and PARACEST DCE-MRI are summarized in Table 7.5.

Table 7.5 Summary of $K_{\text{Trans}}$ from T1 and PARACEST DCE-MRI.

<table>
<thead>
<tr>
<th>DCE-MRI</th>
<th>Contrast agent</th>
<th>$K_{\text{Trans}}$</th>
<th>$\Delta K_{\text{Trans}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Gd-DTPA</td>
<td>17.82</td>
<td>0.031</td>
</tr>
<tr>
<td>T1</td>
<td>Alb-Gd-DTPA</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>PARACEST</td>
<td>Tm-DOTAMGly</td>
<td>164.57</td>
<td>0.105</td>
</tr>
<tr>
<td>PARACEST</td>
<td>Alb-Yb-DOTAMGly</td>
<td>17.32</td>
<td></td>
</tr>
</tbody>
</table>

* define $K_{\text{Trans}} = \frac{\text{ml/min}}{100\text{ml of tumor volume}}$
Figure 7.5 The results from T1 DCE-MRI. MR intensities were measured from the acquired DCE-MRI images. The MR intensities were converted to the concentration of contrast agents, respectively. In this model, whole tumor region was selected to process data.
Figure 7.6 PARACEST DCE-MRI of Albumin-(Yb-DOTAMGly). A) The PARACEST effect (% CEST) was calculated from the “Off-resonance” image and “On-resonance” images. The PARACEST effect was converted to the concentration of contrast agent, LMCA, which is shown in B).
Figure 7.7 PARACEST DCE-MRI of Tm-DOTAMGly. A) The PARACEST effect (% CEST) was calculated from the “Off-resonance” image and “On-resonance” images. The PARACEST effect was converted to the concentration of contrast agent, SMCA, which is shown in B).

Figure 7.7 PARACEST DCE-MRI of Tm-DOTAMGly. A) The PARACEST effect (% CEST) was calculated from the “Off-resonance” image and “On-resonance” images. The PARACEST effect was converted to the concentration of contrast agent, SMCA, which is shown in B).
Figure 7.8 The values of $K_{\text{Trans}}$ from DCE-MRI. The results from $T_1$ DCE-MRI are shown in A) and the results from PARACEST DCE-MRI are shown in B). In this model, the time is assumed as PATLAK Time, which needs to be clarified with additional data and analysis.
4. Discussion

To compare PARACEST DCE-MRI with conventional T₁ DCE-MRI, two different DCE-MRI methods were performed and compared with each other. As preliminary PARACEST DCE-MRI experiments, the results show relatively good relationships between T₁ DCE-MRI and PARACEST DCE-MRI.

But there are several obstacles to perform more reliable and reproducible PARACEST DCE-MRI. Most of all, the sensitivity of contrast agents should be enhanced. The measured concentrations of contrast agents in the tumor are almost overlapped with the sensitivity thresholds of each contrast agent, which is obtained from the calibration curves for Tm-DOTAMGly albumin-(Yb-DOTAMGly). This lower sensitivity causes larger errors in vivo PARACEST DCE-MRI. Also, the purification of SMCA is another issue in contrast agent. The salt such as NaCl is inevitably mixed with SMCA, which causes inaccuracy in the calibration curve of SMCA. In the case of LMCA, the salt can be removed by dialysis so that it has a negligible effect on the calibration curve of LMCA.

In performing PARACEST DCE-MRI, the temperature and the breathing rate monitoring show fluctuations that can affect on motion artifacts and relaxivities of water and contrast agents. To control the temperature, hot air was blown to heat up the mouse model but there’s no cooling method during MR measurements. The more accurate temperature
control is required in the future modification of PARACEST DCE-MRI. Also, the motion artifacts are serious in several cases. For PARACEST DCE-MRI, the acquisition time is as long as 1 minute or more. The long acquisition time magnify the motion artifacts caused by breathing. The breathing rate was usually maintained at less than 30 breaths/min, but the breathing rate usually fluctuated between 60 and 40 and sometimes rapidly increased to 90 immediately after the injection of contrast agents. The breathing rate was controlled by regulating the flow rate of isoflurane from the vaporizer, but it’s not accurate and needs continuous monitoring of breathing rates. Automated responsive control of the flow rate of isoflurane can provide more reliable breathing control than manual control. To minimize the motion artifacts, the body movement caused by breathing should be suppressed. Currently, the taping method was used on the plate equipped with a nose cone for the anesthization. But the taping method has limitations in suppressing the motion of breathing. A muscle relaxant and accompanying utilities can be considered for the minimization of motion artifacts in PARACEST DCE-MRI.

In injecting contrast agents, the injection rate should be considered. Currently the contrast agents are injected manually by measuring time and injection volume. The manual injection rate is irregular and not a continuous injection. Also, by opening and closing the door during MR measurements, the images around the injection time have serious
artifacts. By the use of commercially available non-magnetic automatic syringe injector, this problem can be resolved.

In PARACEST DCE-MRI analysis, several assumptions are made such as $C_{1,p}(t) = C_{2,p}(t)$, setting $e^{t_{kep}}$ as a constant because the influx is usually considered as negligible and $K^{\text{Trans}} \gg \nu_e/(C*\text{patlak}(t))$. $C_{1,p}(t)$ can be difficult to correlate with $C_{2,p}(t)$ during in vivo studies. To resolve this problem, the arterial input function (AIF), $C_{a,1}(t)$ and $C_{a,2}(t)$, should be measured to estimate $C_{1,p}(t)$ and $C_{2,p}(t)$ by the equation of $C_a(t)=C_p(t)*(1-Hct)$. Hct in highly perfused tissue (such as tumor) is known as approximately 0.45 but can be lower 0.30–0.35 or higher. In these experiments, MDA-MB-468 human breast cancer cells were used for $T_1$ DCE-MRI and MCF7 human cancer cells were used for PARACEST DCE-MRI. These two experiments were designed separately and carried out independently. MDA-MB-468 tumor model is known as highly metastatic tumor and MCF tumor model is known as much less metastatic. The characteristics of each tumor models are not characterized completely in this thesis, but two different human breast cancer models are expected to show different pharmacokinetic response to the contrast agents, which is expected to affect on the determination of $K^{\text{Trans}}$ and $\Delta K^{\text{Trans}}$.

$K^{\text{Trans}}$ is larger than $\nu_e/(C*\text{patlak}(t))$ in highly permeable tissue at longer times, so that the end of the DCE-MRI curve should be evaluated, that means Patlak(t) of SMCA and
LMCA may be different because of their physiological response affected by physical size, ionic charges and binding affinities with proteins or lipids in the blood or tissues.

To enhance the availability of the flank tumor model, the tumor should be implanted as low as possible in the flank to minimize the effect of breathing. The image slices for DCE-MRI should include the thigh to collect the AIF from the femoral artery. In many cases, the acquisition of the AIF in small animals is reported to be difficult and marginally successful. Or bending the tail with injection line to the body side can resolve this problem by measuring the tail artery and tumor at the same time.

There are many details to be resolved in PARACEST DCE-MRI to expand the application as the conventional T<sub>1</sub> DCE-MRI, but there are many expected advantages in PARACEST DCE-MRI that T<sub>1</sub> DCE-MRI can not support. According to the difference of lanthanide ions, two different contrast agents can be measured at the same time by adjusting chemical shifts for each of contrast agent. In the future research, the assumptions in PARACEST DCE-MRI model equations should be corrected based on the experimental results and the dual injection and contemporary monitoring of two contrast agents should be accomplished.

However, the first demonstration of PARACEST DCE-MRI of each agent establishes a milestone in the development of this technique.
5. References


Scheme 7.1 Synthesis of DOTAMGly

\[ \text{C}_8\text{H}_{20}\text{N}_4 \text{ BrNO}_3 \]
Exact Mass: 222.98  
Mol. Wt.: 224.05  
4.1 eq. 41mmol, 9.19g

\[ \text{C}_6\text{H}_{10}\text{BrNO}_3 \]
Exact Mass: 172.17  
Mol. Wt.: 172.27  
10 mmol, 1.72g

\[ \text{K}_2\text{CO}_3 \text{ Mol.Wt.:138.21} \]
8eq. 80mmol, 11.06g

\[ \text{Solvent : Distilled Acetonitrile 200ml} \]
Refux 6hrs  
Ar gas environment  
Filter and remove solids.

Concentrate the filtrated solution under reduced pressure (Rot.Evap.)
To remove color, dissolve the product in water and treat with "activated carbon"

Hydrolysis with 1.0N NaOH
Dissolve DOTAM-Gly-OEt in EtOH/Water(100ml/100ml)
Heat flask to 60 °C
Add several mL of 1N-NaOH and check pH
During the hydrolysis, the pH will decrease with time.
Add 1N-NaOH solution to maintain pH at 11.
After verifying that pH is constant,
cool to room temperature.
Acidify with 6N-HCl and adjust to pH 2 ~ 3.
Purify DOTAM-Gly with Sephadex-G10
or
Amberlite reverse phase liquid chromatography

\[ \text{C}_8\text{H}_{40}\text{N}_8\text{O}_{12} \]
Exact Mass: 632.28  
Mol. Wt.: 632.62  
Calculated Product : 6.33g
PARACEST contrast agents have many advantages. Most of all, the selectivity is the first aspect to be counted. By the irradiation of MR frequencies to activate any specific contrast agents in the sample/target tissues, we can measure the expected chemical structural change in the contrast agent, which is affected by micro-environment (e.g. pH), biological interactions (e.g. enzyme activity) or physical/distributional density. But, like other contrast agents, there are some limitations in PARACEST contrast agents. The sensitivity is the main concern for PARACEST contrast agents. Because it’s based on the magnetization transfer by proton exchanges between water and the contrast agent, the sensitivity is relatively lower than $T_1$ or $T_2^*$ contrast agents. It is expected that the sensitivity can be enhanced by the molecular design of contrast agents that have faster exchange and/or slower $T_1$ and $T_2$ relaxation times. The specificity is another issue for PARACEST contrast agents. Saturation of just one PARACEST frequency while avoiding the saturation of another PARACEST frequency or water is critical for selectively detecting one biomarker.
Furthermore, from the perspective of biomedical engineering, the final goal of molecular imaging should be the practical applications in laboratory or clinical fields. To accomplish this goal, new research methodologies will be suggested as tentative projects.
1. Development of Contrast agents

New synthetic pathways have been developed to prepare peptidyl PARACEST MR imaging contrast agents. To introduce the DOTA moiety at the C-terminus of peptides, the solution phase and solid phase synthetic methodologies were demonstrated that have good purity and yield and currently these technical methods are being used to engineer new peptidyl contrast agents.

If the DOTA moiety can be introduced in any sequence of peptides, it will provide immeasurable diversity in the design of peptidyl imaging probes. Also, polymeric/liposomal/dendritic vehicles can be used to load/couple a lot of imaging probes inside/outside the 3D-structural templates. It will generate higher sensitive imaging contrast by the localization of imaging probes. The details are described as follows.

A. Synthesis of DOTA derivatives

Currently, CBZ and BOC protecting groups have been investigated for the orthogonal synthesis of contrast agents. To cleave the CBZ protecting group, substantial loss has been observed during the CBZ cleavage reaction with Et₂AlCl/thioanisole. If an Fmoc group can be introduced to protect the amino group of aminoDOTA and if one of the carboxylic acid groups remains free without protection, the DOTA moiety can be coupled
to any sequence of peptides following conventional Fmoc-synthetic protocols without any serious loss in yield. The expected synthetic pathway is shown in Scheme 8.1.

By the use of any kind of polymeric supports that are compatible with Fmoc-synthetic protocols, the first few amino acids can be loaded on the resin and Fmoc-Gly(DOTA-tBu)-OH can be coupled as one of the amino acids. After removal of the Fmoc protecting group, any sequence of peptides can be elongated from the amino group of aminoDOTA. This approach is expected to provide much higher flexibility in designing and synthesizing of peptidyl contrast agents with higher yield for molecular imaging research.

Any imaging contrast agent that needs a peptidyl ligand as a targeting probe and DOTA or DOTA derivative as a metal chelator, can be developed for MRI, PET and SPECT imaging modalities.

Another structure of aminoDOTA derivatives can be drawn from peptidyl DOTA phosphate derivatives (Scheme 8.2 and 8.3). The lanthanide ions can play a role as a catalyst in cleavage of phosphate ester bond when there are water molecules to help the hydrolysis reaction. The proposed structure of a drug linked peptidyl phosphate DOTA is expected to be stable in physiological environments. When there are specific enzymes to cleave the peptide to disclose one empty ligand for the access of water molecules, the phosphate ester will be cleaved automatically to release the drug linked to the DOTA
moiety. This concept of an enzyme responsive, drug releasing MR contrast agent will provide information such as disease status, enzyme activity and drug releasing efficiency. Through the cleavage of a peptide and subsequent drug release, the water access will be made easier and it will cause the change of relaxivity.

A Trimethyl lock mechanism is expected to be useful for the detection of enzyme activity (Scheme 8.4). The peptide is linked to the phenolic group and the trimethyl lock can be activated by the cleavage of the peptide by a specific enzyme.

The carboxylic acid of Fmoc-Gly(DOTA-tBu)-OH can be derivatized to a thioester for the ligation reaction. Fmoc-DOTA can be applied to the ligation reaction by the introduction of azide or thioester group by transthioesterification (Scheme 8.5). The modified carboxylic group can be used to couple with amino group of peptides. The reaction mechanism and the coupling condition are quite different from conventional Fmoc-synthesis protocol, but it is expected to be helpful to labeling peptides and polymeric/liposomal/dendritic templates.

Another major future direction is to use amino acid ligands other than “Glycine” such as aspartic acid and glutamic acid for the introduction of a carboxylic acid, lysine for an amine group, serine and tyrosine for a hydroxyl group and cystein for a thiol group. The general synthetic pathways for each compound are shown in Scheme 8.6.
**Scheme 8.1** Tentative synthetic pathway of Fmoc-Gly(DOTA-tBu)-OH

* Instead of H₂/Pd-C hydrogenolysis condition, 1 eq. of cyclohexadiene can be used.
Scheme 8.2 Proposed mechanism of enzyme responsive peptidyl phosphate DOTA. The drug linked to the phosphate group will be released by an autocatalytic reaction of the lanthanide and water.
Scheme 8.3 Tentative synthetic scheme of peptidyl phosphate DOTA with doxorubicin labeled as D in the Scheme.

![Scheme 8.3](image-url)
Scheme 8.4 Tentative enzyme responsive MR contrast agent based on the trimethyl lock mechanism.
Scheme 8.5 Tentative synthetic pathway of Fmoc-Gly(DOTA-tBu) derivative for ligation

* A phenyl group is shown in this tentative scheme, but any protecting group for conventional ligation reactions can be introduced as a protecting group for this thioester.
Scheme 8.6 Tentative synthetic pathway of Fmoc-AA(DOTA-tBu)-OH

R represents the side chain of the amino acid.
P represents the protecting groups of the carboxylic acid
B. Polymeric / Dendrimeric Templates

To enhance the sensitivity to achieve at least a 5% change in image contrast, it is important to concentrate the contrast agents in a local region. Considering the sensitivity of small molecular PARACEST contrast agents, for example, Tm-DOTAMGly can be detected as low as 2.5 mM. If the small molecular contrast agent was loaded inside liposomes or coupled to the surface of polymeric or dendritic templates with higher concentration than the threshold of minimal detection sensitivity, much lower polymeric/liposomal/dendrimeric contrast agents can be detected in one voxel. It was thought that at least 1~10 mM of small molecular contrast agents are required to be detected by MRI. But, considering a recent report about LIPOCEST agents, the liposome contrast agent can be detected at concentrations as low as 90 pico molar (pM) concentration. The sensitivity of LIPOCEST shows extremely low concentration limit, which shows the possibility of detection of liposome contrast agent in a voxel. If 4500 particles of LIPOCEST agents can be localized in 100um x 100um x 100um volume, hypothetically it can be detected with at least 5% threshold in a voxel. Usual nano-sized polymeric/liposomal particles are 100nm size in diameter, so that the voxel will show 5% contrast change when 0.45% of voxel is filled with LIPOCEST agents. Also, considering several self-assembling nano particles, they have molecular weights of 100,000 and there
are approximately 1000 functional groups to be coupled with the small molecular contrast agents. By the localization of contrast agents with the help of polymeric/liposomal/dendritic templates, the sensitivity of PARACEST contrast agents can be highly enhanced compared to the small contrast agent itself.

2. Applications for diagnostic/clinical research

Enzyme activity can be detected by PARACEST contrast agents by the enzymatic bioconversion of chemical structures. Also, the peptidyl-DOTA structure can be elongated on the polymeric supports as demonstrated in this thesis. The polymeric supports can be substituted by any supports with functional groups on the surface. The glasses or polymer plates or wells can be an alternative synthetic support by the surface modification. For example, the hydroxyl group of a glass surface and the carboxylic/amino/hydroxyl groups of polymers are eligible for chemical modifications. The DOTA moiety is expected to be loaded on the functional groups on those supports.

By following conventional Fmoc peptide synthesis protocols, any kind of peptidyl or peptide mimetic sequences will be synthesized. Also, many different targeting probes can be loaded in each well to monitor several enzyme activities simultaneously. This
technique is based on the “Lab-on-a-Chip” or “Peptide-Array Chip”.

Currently UV/fluorescence based contrast agents are mainly used for the detection of enzyme activities. But it needs laborious and time consuming pre-treatment of each sample to acquire the repeatable results from the measurements. The main disadvantage of optical detection methods is the interference of light by particles. To remove the error by interferences, any solids or chromophores should be removed during the pre-treatment step. The PARACEST MR imaging technique can provide the solutions from the aspect of pre-treatment time and labors. Also, high throughput screening is applicable with this method, which is more practical and convenient with the increasing number of samples.

Considering the sensitivity of PARACEST MR contrast agents, 20 μmole/100μl/well will show 200 mM concentrations. This means that only 1 μmole of contrast agent will show 10 mM concentration in a 100 μl well. To facilitate handling, the peptide-DOTA loaded resin can be immobilized in a well. In this case, only 5 mg of resin is required to show 10 mM concentration by use of 0.2 mmole/g of substitution ratio. The plates can be used for high throughput screening of specific enzyme activity. The schematic assay kit is shown in Figure 8.1.
Figure 8.1 Schematic figure of a diagnostic enzyme assay kit. In each well, different peptide-DOTA agents can be loaded to detect different enzyme activities. For the kinetics studies, different concentration of peptide-DOTA can be loaded in a row or column. A is the case that each well is filled with the peptide-DOTA loaded resin and capped with a semi permeable membrane. B is the case that peptide-DOTA is implanted on the surface of each well.
3. Detection of endopeptidase activity

An endopeptidase-targeting peptidyl contrast agent was tested during the development of this thesis. For this study, MMP-9 and MMP-2 enzymes were chosen as endopeptidases. Endopeptidases cleave the middle of peptide sequences and the substrates of MMP-2 and MMP-9 are listed in Table 8.1. The hypothesis is that enzymatic cleavage will generate a new PARACEST effect from the amine proton, or cause the disappearance of an existing PARACEST effect from amide protons. To verify the hypothesis, MMP-2 targeting peptides were chosen from the Table 8.1 and the sequences were Ac-PLG-LLAA, Ac-PLG-MWSG, Ac-DDDPLG-MWSR, Ac-PLG-MWSR (kcat/KM = 1.3 x 10^4) and SGAVRW-LLTA (kcat/KM = 2 x 10^5). Each peptide sequence was coupled to DOTA at the C-terminus and measured to verify the PARACEST peaks. But, none of peptidyl contrast agents showed meaningful PARACEST peaks. To resolve the problems in the future research for the development of endopeptidase responsive PARACEST MR contrast agents, several points should be considered from molecular design to in vitro enzymatic evaluation.

Firstly, in the designing of molecular structure for MMP targeting peptide-DOTA, the length of peptide should be considered. From computer aided molecular modeling studies, 5~7 amino acid residues can make one turn to make the N-terminus approach to the core
lanthanide ion chelated in the core of DOTA ligand. The PARACEST peak is expected to be generated from the acylamide proton at the N-terminus of peptide or from the amine proton at the N-terminus after enzymatic cleavage. The former structure is expected to show one less PARACEST peak compared with the product generated after the enzymatic cleavage, so it can be classified as a “Switch Off” contrast agent. The latter one is expected to show one more PARACEST peak compared with the product generated after the enzymatic cleavage, so it can be classified as a “Switch On” contrast agent.

For the design of a “Switch Off” contrast agent, the N-terminus needs to be modified by an additional functional group. The introduction of a carbamic acid group can provide better functionality than aspartic acid, which was tested with DDDPLG-MWSR-DOTA.

The schematic structure is shown in Figure 8.2 (A). To make the synthesis steps short, the side chain of lysine can be modified by chloroformate. The carboxylic acid moiety is expected to form a ligand with lanthanide ion and the proton of carbamic acid is expected to generate a PARACEST effect. After the enzymatic cleavage, the fragmented N-terminus portion will be separated from the contrast agent to switch off the PARACEST signal.

For the design of a “Switch On” contrast agent, the peptide needs to be modified at the C-terminus. The N-terminus of the fragmented peptide must come back to lanthanide core
ion to generate a PARACEST signal from amine protons. To control the exact length of the peptide for the spontaneous turning back of the N-terminus to the lanthanide ion core, the C-terminus can be modified by any amino acid with hydrophilic side chains as shown in Figure 8.2 (B). The hydrophobic side chains can cause intermolecular interaction in water.

The balance of hydrophilicity and hydrophobicity is an important factor in attracting the N-terminus to the lanthanide ion core. The intermolecular aggregation can happen and inhibit the turning back of the N-terminus of peptide to lanthanide core ion. Even though the computer aided molecular modeling study showed turning back of the N-terminus to lanthanide ion, in experiments, it does not show any meaningful PARACEST peak generated from the acylamide proton at the N-terminus of the peptide. Also, the amino group can be positively charged and has a very hydrophilic property. The driving force of turning back the peptide to the lanthanide ion could be ionic interactions between the amino group and the lanthanide core ion. But, according to thermodynamic considerations, it is hardly possible to create an interaction between the N-terminus and the lanthanide core ion. The positively charged amino group can interact with water molecules more easily by forming hydrogen bonds or dipole-dipole interactions, relative to forming an interaction with the positively charged lanthanide ion.
Secondly, the chelation step is another issue that is related with peptide sequences. Currently, lanthanide chlorides or lanthanide triflates are used for the chelation of lanthanides in the ligands. The lanthanide chlorides are used in water or buffer solution without any organic solvents except small amounts of ethanol or methanol. The lanthanide triflates are used in organic solvents such as ethanol, methanol, acetonitrile, acetone and small amounts of water are allowed to mix with organic solvents.

If the peptide has hydrophobic characteristics, it does not dissolve in water or organic solvents within pH range of chelation (pH 6 ~ 8). The lower solubility of hydrophobic peptides has caused a chelation problem in all cases. To overcome this solubility problem, the peptide sequences have been modified to increase the solubility in water. The aspartic acids were elongated at the N-terminus to enhance water solubility, but after 3 aspartic acids were coupled, the solubility was not enhanced so much. The peptide-DOTA was aggregated during the chelation step. The elongation of more aspartic/glutamic acid residues should be helpful to overcome the solubility issue but the number of aspartic/glutamic acid residues should be optimized.

To detect the endopeptidase activity, the peptide-DOTA structure needs to be designed differently from the exopeptidase targeting peptide-DOTA structures. For the future approach to detect endopeptidase activity, tentative structures of contrast agents are
proposed in Figure 8.2 (C) and (D). By the capping the 9th chelation site of lanthanide ion with the intramolecular cross-linked peptide, the water accessibility can be minimized as shown in Figure 8.2 (C). By the enzymatic cleavage, the 9th chelation site is expected to form a ligand with water to show a PARACEST effect from metal bound water. But the solubility issue in chelation step is remained unsolved. Another possible structure is to introduce a PEG moiety at the end of the N-terminus to enhance water solubility as shown in Figure 8.2 (D). Conventionally polyethylene glycol (PEG MW 5000) is used for the surface modification of nanoparticles to enhance solubility and inhibit coagulation in the hemocirculation system. With PEG modification, the solubility of contrast agent will be highly enhanced and overcome the problem in chelation step.
Figure 8.2 Schematic figures of endopeptidase detecting PARACEST contrast agents

(A) Introduction of carboxylic acid at the N-terminus of peptide

(B) Introduction of hydrophilic amino acids at the C-terminus of peptide

(C) Capping by cross-linked peptide

(D) Pegylate peptide-DOTA
Table 8.1 MMP’s substrate peptide sequences

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Abu: L-a-Aminobutyric acid, β-cy: β-cyclohexyl, Cha: L-cyclohexylalanin, Dpa: 3-(2,4-dinitrophenyl)-L-2,3-diaminopropionic acid or N-3-(2,4-dinitrophenyl)-L-α,β-diaminopropionyl, FTC: Fluorescein-Thiocarbamoyl, MCA: (7-Methoxycoumarin-4-yl)acetyl, Nva: L-Norvaline, SCH: SCHCO (CH2CH(CH3)2), 2-Mercapto-4-methylpentanoyl
Appendix

A1. Michaelis-Menten kinetics versus CEST concentration calibration

The enzymatic catalysis of a substrate is a multistep process involving chemical reactions and intermolecular associations and dissociations. The theory of Michaelis-Menten kinetics simplifies this process by assuming that the chemical reaction rate is much slower than the rate of dissociation of the product with the enzyme ($k_2 \ll k_3$), and the association of the product and enzyme is negligible ($k_{-3} \approx 0$). In most cases the chemical reaction rate is also slower than the dissociation rate of the substrate with the enzyme ($k_2 \ll k_{-1}$).

$$[E] + [S] \xrightleftharpoons[k_{-1}]{k_1} [ES] \xrightarrow{k_3} [E] + [P] \xrightarrow{k_{-3}} [EP]$$

Many other assumptions are made by the relatively simplistic Michaelis-Menten kinetics theory, such as the absence of allosteric effects, so that the accuracy of experimental results must be evaluated on a case-by-case basis. Yet these assumptions allow the association of the substrate with the enzyme to be analyzed independently from the rate of the chemical reaction.

The chemical reaction rate, $k_{\text{cat}}$, is determined from the maximum initial reaction velocity
when the enzyme is saturated with substrate, $V_{\text{max}}$ (equation A1.1). The Michaelis constant, $K_M$, represents the substrate concentration at half saturation that can achieve half of $V_{\text{max}}$. In practice, selecting substrate concentrations that ensure full saturation and half saturation of the enzyme is daunting, so that $V_{\text{max}}$ and $K_M$ are typically determined through graphical analyses or parameter estimations of the initial reaction velocities, $v_i$, determined over a range of substrate concentrations, $[S]$ at a single enzyme concentration $[E]$ (equation A1.2). Molecular imaging contrast agents that act as substrates may be used to analyze Michaelis-Menten kinetics of an enzyme by measuring the initial reaction velocities, either through detection of the disappearance of the substrate and/or the detection of the appearance of the product.

\[ k_{\text{cat}} = \frac{V_{\text{max}}}{[E]} \quad \text{(Eq. A1.1)} \]

\[ v_i = \frac{V_{\text{max}} [S]}{(K_M + [S])} \quad \text{(Eq. A1.2)} \]

A Hanes plot is a rapid graphical analysis method that provides relatively accurate results (Table A1.1). The Hanes plot is less sensitive to measurement inaccuracies at low substrate concentrations that are problematic for the Lineweaver-Burk plot, which uses the inverse of substrate concentrations. The Hanes plot uses one independent variable
on the abscissa, which allows for easier interpretation of errors than the Eadie-Hofstee plot that includes dependent variables on both sides of the linear relationship.6

Table A1.1  A comparison of graphical analysis methods to evaluate Michaelis-Menten kinetics parameters.

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<th>Analysis Method</th>
<th>Equation</th>
<th>Graphical Plot</th>
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This experimental design can be extended to simultaneously analyze two enzymes with two molecular imaging substrates. If each substrate is specifically cleaved by only one enzyme, the Michaelis-Menten kinetics analysis is straightforward. However, proteases are often promiscuous, and cleave substrates of other proteases. To deconvolute the enzymatic activities of two promiscuous proteases, an unresponsive molecular imaging contrast agent must be included to account for total substrate concentrations (equation A1.3 and A1.4). This analysis method can be accomplished by measuring the concentrations of the unresponsive agent and the substrates and/or products at a single time point, which is critical for molecular imaging modalities with slow temporal resolution such as MRI. In addition, detection of the unresponsive agent accounts for fluctuations in the total concentrations of contrast agents due to pharmacokinetics, assuming that the pharmacokinetics of the contrast agents is equal (e.g. are covalently linked). This analysis method can be extended to simultaneously evaluate three or more enzymes with three or more enzyme-responsive contrast agents, as long as an unresponsive contrast agent is also included.

\[
[E1] = \frac{1}{[k_{S1-E1} k_{ratio} t]} \left[ \left( \frac{k_{S1-E2}}{k_{S2-E2}} \right) \ln\{1-f_{p2}\} - \ln\{1-f_{p1}\} \right] \quad \text{(Eq. A1.3)}
\]
\[
[\text{E2}] = \frac{1}{k_{\text{S2-E2}} k_{\text{ratio}} t} \left[ \ln \{1-f_{\text{P1}}\} - \ln \{1-f_{\text{P2}}\} \right] \quad \text{(Eq. A1.4)}
\]

Definitions for equations A1.3 and A1.4:

- [P1]: concentration of product #1 (substrate #1 after reaction)
- [P2]: concentration of product #2 (substrate #2 after reaction)
- [S1]: concentration of substrate #1 (before reaction)
- [S2]: concentration of substrate #2 (before reaction)
- [SP1]: total concentration of substrate #1 and product #1
- [SP2]: total concentration of substrate #2 and product #2
- [SP3]: total concentration of unresponsive substrate #3 (concentration of product of substrate #3 = 0)
- [E1]: total concentration of enzyme #1
- [E2]: total concentration of enzyme #2
- t: time
- \(k_{\text{S1-E1}}\): rate of chemical reaction of substrate #1 with enzyme #1
- \(k_{\text{S1-E2}}\): rate of chemical reaction of substrate #1 with enzyme #2
- \(k_{\text{S2-E1}}\): rate of chemical reaction of substrate #2 with enzyme #1
- \(k_{\text{S2-E2}}\): rate of chemical reaction of substrate #2 with enzyme #2
- \(k_{\text{ratio}} = 1 - \left( \frac{k_{\text{S2-E1}} k_{\text{S1-E2}}}{k_{\text{S1-E1}} k_{\text{S2-E2}}} \right) \)
- \(f_{\text{P1}} = \frac{[P1]}{[SP1]}\)
- \(f_{\text{P2}} = \frac{[P2]}{[SP2]}\)

Equations A1.3 and A1.4 are derived from the concentration of product at specific times after the reaction is initiated:

\[
[P1] = SP1 \left[ 1 - \exp\{-t (k_{\text{S1-E1}}[E1] + k_{\text{S1-E2}}[E2])\} \right] \quad \text{(Eq. A1.5)}
\]

\[
[P2] = SP2 \left[ 1 - \exp\{-t (k_{\text{S2-E1}}[E1] + k_{\text{S2-E2}}[E2])\} \right] \quad \text{(Eq. A1.6)}
\]
The derivation of equations A1.3 and A1.4 from equations A1.5 and A1.6 is based on the following assumptions:

- \([\text{SP1}] = [\text{SP2}] = [\text{SP3}]\) (i.e., all substrates are equally delivered to the site of interest)
- \(k_{S1-E1}, k_{S1-E2}, k_{S2-E1},\) and \(k_{S2-E2}\) are known from previous assays under conditions that are identical to the current experiment
- \([\text{P1}], [\text{P2}],\) and \([\text{SP3}]\) can be measured. Alternatively, \([\text{S1}]\) and/or \([\text{S2}]\) can be measured because \([\text{P1}] = [\text{SP3}] - [\text{S1}]\) and \([\text{P2}] = [\text{SP3}] - [\text{S2}]\)
- This analysis must be conducted before all products have been formed, so that \(f_{P1}\) and \(f_{P2}\) are less than 1.
- The ratios of the chemical reaction rates for \(S2\) vs. \(S1\) must not be the same for \(E1\) and \(E2\), so that \(k_{S2-E1} / k_{S1-E1} \neq k_{S2-E2} / k_{S1-E2}\). Otherwise, \(k_{\text{ratio}} = 0\) and the analysis fails.

Reference

A2. MEROPS Database

The MEROPS database is an on-line information resource for peptidases, substrates and inhibitors. A search of the MEROPS database identified 242 unique proteases that cleave a relatively large blocking group at the N- or C-terminus of a specific peptide sequence (Table A2.1). This list of proteases was further stratified to identify 88 human proteases, 30 non-human vertebrate proteases, 96 bacterial and parasitic proteases, 25 plant proteases, and 3 proteases of unidentifiable origin. The list of human proteases was further stratified to identify 18 cysteine proteases, 12 metalloproteases, 56 serine proteases, 2 threonine proteases.

A survey of the MEROPS database revealed that 12.3% of the currently identified human proteasome consists of exoproteases that can accommodate substrates with relatively bulky N- or C-terminal groups (Table A2.2). This result is a conservative estimate, as all identified human proteases may not yet have been studied with substrates that incorporate bulky N- or C-terminal groups. This human ‘exoproteasome’ consists of a high percentage of serine proteases and a low percentage of metalloproteases and aspartic proteases, relative to the distribution of these catalytic mechanisms in the human proteasome. Cysteine proteases are equally represented in the ‘exoproteasome’ relative to the proteasome. This different distribution of the ‘exoproteasome’ and proteasome
may reflect the relative abilities of protease types to accommodate a bulky peptide terminal group while still maintaining catalytic function. This conjecture is supported by visual inspection of all available three-dimensional structures of human proteases, which revealed a deep binding pocket in almost all structures of aspartic proteases, a shallow binding pocket in serine proteases, and more variable binding pocket structures in cysteine proteases and metalloproteases. More experimental studies of intermolecular interactions between specific peptidyl-DOTA contrast media and specific proteases are required to validate the general conclusions of this analysis of the MEROPS database. A survey of the MEROPS database indicated that this new type of contrast media may be applied to detect a broad distribution of the human proteasome, and therefore may constitute a new platform technology for molecular imaging.
<table>
<thead>
<tr>
<th>Type</th>
<th>Protease</th>
<th>Biomedical role, other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>calpain-2</td>
<td>Drug target for stroke and neural injuries</td>
</tr>
<tr>
<td></td>
<td>caspase-1</td>
<td>Apoptosis, response to infection</td>
</tr>
<tr>
<td></td>
<td>caspase-3</td>
<td>Apoptosis, response to infection</td>
</tr>
<tr>
<td></td>
<td>cathepsin B</td>
<td>Potential drug target for cancer (e.g. Bervar et al., 2003), proposed to be responsible for in acute pancreatitis</td>
</tr>
<tr>
<td></td>
<td>cathepsin F</td>
<td>Atherosclerosis lesions, macrophages</td>
</tr>
<tr>
<td></td>
<td>cathepsin H</td>
<td>Lysosomal proteolysis, atherogenesis by modifying LDL</td>
</tr>
<tr>
<td></td>
<td>cathepsin L</td>
<td>Endopeptidase activity in lysosomal proteolysis</td>
</tr>
<tr>
<td></td>
<td>peptidase 2</td>
<td>Cathepsin L-like peptidase 2</td>
</tr>
<tr>
<td></td>
<td>cathepsin O</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>cathepsin S</td>
<td>Likely roles are in lysosomal proteolysis, extracellular proteolysis including elastin degradation, and the MHC class II immune response. Cathespin S has been proposed to be involved in angiogenesis (Shi et al., 2003) and atherogenesis (Sukhova et al., 20</td>
</tr>
<tr>
<td></td>
<td>cathepsin V</td>
<td>In human thymic epithelial cells, suggest potential involvement in the immunopathogenesis of myasthenia gravis</td>
</tr>
<tr>
<td></td>
<td>cathepsin W</td>
<td>Unknown, mainly in endoplasmic reticulum</td>
</tr>
<tr>
<td></td>
<td>cathepsin X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>peptidase I</td>
<td>Dipeptidyl-peptidase I. Papillon-Lefevre syndrome, Haim-Munk syndrome target for sepsis therapy</td>
</tr>
<tr>
<td></td>
<td>legumain (chordate)</td>
<td>Cancer</td>
</tr>
<tr>
<td></td>
<td>pyroglutamyl-peptidase I (vertebrate)</td>
<td>Membrane and extracellular protein that processes hormone TRH for neuroregulation</td>
</tr>
<tr>
<td></td>
<td>ubiquitin-specific peptidase 5</td>
<td>Autophagy (programmed cell death)</td>
</tr>
<tr>
<td>Metallo</td>
<td>aminopeptidase A</td>
<td>Putative target for angiogenesis in cancer, putative central antihypertensive agents</td>
</tr>
<tr>
<td></td>
<td>aminopeptidase B</td>
<td>Development</td>
</tr>
<tr>
<td></td>
<td>aminopeptidase N</td>
<td>Cancer, atherosclerosis, rheumatoid arthritis</td>
</tr>
<tr>
<td>Aminopeptidase PILS</td>
<td>Putative role in angiogenesis</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td>AMZ1 g.p. (Homo sapiens) and similar</td>
<td>Role unknown</td>
<td></td>
</tr>
<tr>
<td>archelysin (eukaryote)</td>
<td>Newly discovered, predominant in heart, function unknown</td>
<td></td>
</tr>
<tr>
<td>cystinyl aminopeptidase</td>
<td>Development</td>
<td></td>
</tr>
<tr>
<td>cytosol alanyl aminopeptidase</td>
<td>Development</td>
<td></td>
</tr>
<tr>
<td>dipeptidyl-peptidase III</td>
<td>Abundant, but not sure about role</td>
<td></td>
</tr>
<tr>
<td>leucyl aminopeptidase (animal)</td>
<td>Abundant, but not sure about role</td>
<td></td>
</tr>
<tr>
<td>leukotriene A4 hydrolase</td>
<td>Cancer</td>
<td></td>
</tr>
<tr>
<td>pyroglutamyl-peptidase II</td>
<td>Membrane and extracellular protein that processes hormone TRH for neuroregulation</td>
<td></td>
</tr>
<tr>
<td><strong>Serine Protease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acrosin</td>
<td>Sperm activity</td>
<td></td>
</tr>
<tr>
<td>acylaminoacyl-peptidase</td>
<td>General degradation</td>
<td></td>
</tr>
<tr>
<td>cathepsin G</td>
<td>Bactericidal</td>
<td></td>
</tr>
<tr>
<td>cationic trypsin (Homo sapiens-type)</td>
<td>Pancreatitis</td>
<td></td>
</tr>
<tr>
<td>chymase (human-type)</td>
<td>Possible drug target in immune-related diseases of lung and possible taget for vascular disease</td>
<td></td>
</tr>
<tr>
<td>chymotrypsin B</td>
<td>Digestion</td>
<td></td>
</tr>
<tr>
<td>chymotrypsin C</td>
<td>Digestion</td>
<td></td>
</tr>
<tr>
<td>coagulation factor IXa</td>
<td>Hemophilia B</td>
<td></td>
</tr>
<tr>
<td>coagulation factor Xa</td>
<td>Converts prothrombin to thrombin, so involved in thrombosis</td>
<td></td>
</tr>
<tr>
<td>coagulation factor XIa</td>
<td>Blood coagulation</td>
<td></td>
</tr>
<tr>
<td>coagulation factor XIIa</td>
<td>First component of intrinsic pathway of blood coagulation in mammals, Sotos syndrome, Hageman factor</td>
<td></td>
</tr>
<tr>
<td>complement component 2</td>
<td>Immune defense</td>
<td></td>
</tr>
<tr>
<td>Enzyme Name</td>
<td>Function/Role</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Dipeptidyl-peptidase 8</td>
<td>Tissue remodeling</td>
<td></td>
</tr>
<tr>
<td>Dipeptidyl-peptidase 9</td>
<td>Tissue remodeling</td>
<td></td>
</tr>
<tr>
<td>Dipeptidyl-peptidase II</td>
<td>No specific role</td>
<td></td>
</tr>
<tr>
<td>Dipeptidyl-peptidase IV</td>
<td>Type II diabetes, numerous biological functions in eukaryotes, including involvement in T-cell activation, cell adhesion, digestion of proline containing peptides in the kidney and intestines, HIV infection and apoptosis, and regulation of tumorigenicity in certain melanoma cells</td>
<td></td>
</tr>
<tr>
<td>Enteropeptidase</td>
<td>Possible role in acute pancreatitis</td>
<td></td>
</tr>
<tr>
<td>Fibroblast activation protein</td>
<td>&quot;seprase&quot;, colorectal cancer</td>
<td></td>
</tr>
<tr>
<td>Protein Alpha Subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granzyme A</td>
<td>Targeted cell apoptosis</td>
<td></td>
</tr>
<tr>
<td>Hepsin</td>
<td>Prostate and ovarian cancers</td>
<td></td>
</tr>
<tr>
<td>Kallikrein hK1</td>
<td>Inflammation and blood pressure</td>
<td></td>
</tr>
<tr>
<td>Kallikrein hK11</td>
<td>Ovarian cancer</td>
<td></td>
</tr>
<tr>
<td>Matriptase</td>
<td>Prostate cancer</td>
<td></td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>Drug target for emphysema, cystic fibrosis (Martin et al., 2006), adult respiratory distress syndrome, rheumatoid arthritis and other diseases</td>
<td></td>
</tr>
<tr>
<td>Neuropsin</td>
<td>Role in limiting neuronal hyperexcitability induced by epileptogenic insult, controlling pathogenic events in the hippocampus, and thus neuropsin is a potential drug target for epilepsy, strongly over-expressed in cervical and ovarian carcinomas</td>
<td></td>
</tr>
<tr>
<td>Neurosin</td>
<td>Key role in immune-mediated demyelination, in Multiple Sclerosis</td>
<td></td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>Drug target for acute pancreatitis</td>
<td></td>
</tr>
<tr>
<td>PACE4 proprotein convertase</td>
<td>Processes proinsulin and proenzymes involved in many pathologies</td>
<td></td>
</tr>
<tr>
<td>Pancreatic elastase</td>
<td>Drug target for acute pancreatitis</td>
<td></td>
</tr>
<tr>
<td>Pancreatic elastase II</td>
<td>Drug target for acute pancreatitis</td>
<td></td>
</tr>
<tr>
<td>Serine Protease</td>
<td>Function</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>pancreatic endopeptidase E</td>
<td>4-6% of enzymes in pancreas</td>
<td></td>
</tr>
<tr>
<td>plasma kallikrein</td>
<td>Liberates bradykinin from kininogen. Also plays other roles in blood coagulation and inflammation</td>
<td></td>
</tr>
<tr>
<td>plasmin</td>
<td>Degrades fibrin clots, many other potential roles</td>
<td></td>
</tr>
<tr>
<td>prolyl oligopeptidase</td>
<td>General cystolic degradation</td>
<td></td>
</tr>
<tr>
<td>proprotein convertase 1</td>
<td>Processes proinsulin and proenzymes involved in many pathologies</td>
<td></td>
</tr>
<tr>
<td>proprotein convertase 2</td>
<td>Processes proinsulin and proenzymes involved in many pathologies</td>
<td></td>
</tr>
<tr>
<td>proprotein convertase 4</td>
<td>Processes proinsulin and proenzymes involved in many pathologies</td>
<td></td>
</tr>
<tr>
<td>prostasin</td>
<td>Sodium channel activating protease in epithelial lining - Cystic Fibrosis</td>
<td></td>
</tr>
<tr>
<td>protein C (activated)</td>
<td>Antithrombotic, antiinflammatory serine protease. It proteolytically destroys active coagulation factors Va and VIIIa, thus interfering in thrombin activation.</td>
<td></td>
</tr>
<tr>
<td>stratum corneum chymotryptic enzyme</td>
<td>Atopic dermatitis</td>
<td></td>
</tr>
<tr>
<td>testisin</td>
<td>Ovarian cancer</td>
<td></td>
</tr>
<tr>
<td>thrombin</td>
<td>Causes clotting of blood by limited proteolysis of fibrinogen, Thrombosis is the most common cause of death in the industrialized world and, whether through venous thromboembolism, myocardial infarction or stroke, ultimately involves the inappropriate activity of thrombin (Huntington &amp; Baglin, 2003).</td>
<td></td>
</tr>
<tr>
<td>t-plasminogen activator</td>
<td>Thrombolytic agent, may also be involved in MMP9 activation</td>
<td></td>
</tr>
<tr>
<td>tripeptidyl-peptidase I</td>
<td>Lysosomal degradation</td>
<td></td>
</tr>
<tr>
<td>tripeptidyl-peptidase II</td>
<td>General cystolic degradation</td>
<td></td>
</tr>
<tr>
<td>trypsin I</td>
<td>Digestion</td>
<td></td>
</tr>
<tr>
<td>trypsin-2 (human-type)</td>
<td>Pancreatitis</td>
<td></td>
</tr>
<tr>
<td>tryptase alpha</td>
<td>Drug target for asthma and antigen-induced bronchoconstriction</td>
<td></td>
</tr>
<tr>
<td>Serine Protease</td>
<td>Trypsin beta</td>
<td>Contained in mast cells, involved in several functions including allergic reactions</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>u-plasminogen activator</td>
<td>Thrombolytic agent and prognostic marker for tumors - activates MMP2 and MMP9 (breast cancer)</td>
</tr>
<tr>
<td>Threonine Protease</td>
<td>γ-glutamyltransferase I (mammalian)</td>
<td>Glutathione synthesis</td>
</tr>
<tr>
<td>Threonine Protease</td>
<td>glycosylasparaginase precursor</td>
<td>Aspartylglycosaminuria</td>
</tr>
</tbody>
</table>