BIODEGRADABLE MACROMOLECULAR CONTRAST AGENTS
FOR MAGNETIC RESONANCE IMAGING

by

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ABSTRACT

Magnetic resonance imaging (MRI) is a powerful tool for diagnostic imaging. Low molecular weight paramagnetic Gd(III) complexes are often used to enhance the depiction of disease morphology or functionality. Although safe, these complexes have poor pharmacokinetic performance limiting their application when selective enhancement of the blood pool is needed. Macromolecular Gd(III) complexes have demonstrated significantly higher contrast enhancement of the vasculature because their large size limits their clearance and diffusion from the blood pool into surrounding tissue. Macromolecular Gd(III) complexes have shown superior efficacy over clinically approved contrast agents in the noninvasive detection of a wide range of disease in preclinical studies. Unfortunately, the prolonged circulation of previously designed macromolecular Gd(III) complexes results in the metabolic release and subsequent tissue uptake of highly toxic Gd(III) ions. A promising alternative to this challenge is the development of biodegradable polydisulfide Gd(III) complexes. Initially, these polymers would circulate in the vasculature giving the efficacy of a macromolecular contrast agent, but over time would be gradually degraded via the thiol-disulfide exchange reaction yielding small Gd(III) complexes that can readily be cleared. Specifically, the work undertaken in this project was to modify the degradation of polydisulfide Gd(III) complexes by conjugating poly(ethylene glycol) (PEG) around the disulfide bond of poly(GdDTPA-co-L-cystine) (GDCP) yielding PEG-GDCP. PEG-GDCP was
synthesized in a variety of molecular weights, PEG grafting degrees, and PEG sizes. In vitro, these agents showed degradation to a thiol, which was dependent on the type of PEG grafting. In vivo, PEG-GDCP showed excellent enhancement of the blood pool of mice compared to a clinical control, Gd(DTPA-BMA), including very small vasculature. One agent tested, PEG_{1000}-GDCP, was selected as a lead agent for animal tissue studies and MR angiography (MRA) in rats. Compared to its precursor GDCP, PEG-GDCP had a higher plasma concentration at initial time points, sufficient for an MR examination, but after 10 days had minimal tissue accumulation of Gd(III) in all major organs. MRA results related closely to those determined by Gd(III) plasma concentration; PEG-GDCP gave excellent enhancement in a high-resolution 30 min MRA protocol. Because of excellent imaging qualities and minimal long-term Gd(III) accumulations, PEG-GDCP is promising for further research.
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CHAPTER 1

INTRODUCTION

Magnetic resonance imaging (MRI) is a powerful imaging modality for diagnostic radiology and biomedical research. MR images are produced based on the difference between proton relaxation rates of different tissue, and of diseased versus healthy tissue. MRI is largely used for morphological imaging but the technique can also produce valuable functional and quantitative data. MRI has several distinct advantages when compared to other diagnostic imaging modalities in a clinical situation. MRI does not require the use of a radioactive tracer or X-rays unlike other imaging techniques, such as gamma scintigraphy, X-ray computer tomography (CT), single photon emission computed tomography (SPECT), or positron emission tomography (PET). The temporal resolution between subsequent image acquisition and the spatial resolution within an image are relatively high compared to other imaging modalities. Also, unlike optical imaging techniques, there is no depth limitation meaning that the maximum resolution in the object being imaged is consistent at any point in the object. In general, MRI provides sensitive detection of soft-tissue diseases and is an essential method for the detection and characterization of many diseases, including, among others, cancer; abnormalities of the central nervous, cardiovascular, and musculoskeletal system; and dysfunctions of the liver, kidneys, and spleen (1,2).
Magnetic Resonance Imaging

Magnetization and Magnetic Resonance

MR images are produced when nuclei having intrinsic spin angular momentum interact with a magnetic field (3). Subatomic particles behave quantummechanically, but vector models can also accurately describe MRI since MRI deals with the collective effect of a large number of nuclei. Nuclei of interest in biological systems, such as $^1$H, $^{19}$F, and $^{31}$P, all have spin angular momentum, $\vec{J}$ (4). As is the case for most clinical MRI exams, the proton, specifically the water proton, will be considered.

A proton possesses a polar magnetic moment, represented by the vector quantity $\vec{\mu}$, because the hydrogen nucleus is positively charged and spinning. The spin angular momentum vector is proportional to the magnetic moment vector and is related by eq 1:

$$\vec{\mu} = \gamma \vec{J}$$  [1]

where $\gamma$, the gyromagnetic ratio, is the proportionality constant; the higher the value of $\gamma$, the larger the nuclear magnetic moment in a magnetic field (3).

The magnetic moment vector $\vec{\mu}$ will be oriented at an angle with respect to the static magnetic field $\vec{B}_0$. Since an individual proton is rotating about its axis, it will also have a precession about the main magnetic field. The frequency of the precession is proportional to both the gyromagnetic ratio and the magnetic field strength. This is expressed by the Larmor equation (eq 2), where $\omega_L$ is the Larmor frequency (MHz):

$$\omega_L = -\gamma B_0$$  [2]
Figure 1 shows the schematic relationship between the static magnetic field, the dipolar magnetic moment, and the Larmor frequency (4).

Since the detected NMR signal is produced from the collective effect of many nuclei, a macroscopic magnetization vector $\vec{M}$ is employed. A vector sum is used to describe the total effect of all microscopic magnetic moments, such that

$$\vec{M} = \sum_{n=1}^{N_s} \vec{\mu}_n$$  \[3\]

where $\vec{\mu}_n$ represents the $n$th nuclear spin in an images sample of $N_s$ spins.

In the absence of an external magnetic field $\vec{M} = 0$. Quantum theory can be used to describe the effect of external magnetization on a spin. When a proton is placed in a static magnetic field $\vec{B}_0$, its spin state is split with the Zeemann interaction potential (eq 4)

$$E = -\vec{\mu} \cdot \vec{B}_0 = -\gamma \hbar B_0 I_z$$  \[4\]

The water proton has two possible energy states since $I = \frac{1}{2}$. The difference between the two energy states is proportional to the magnetic field strength $B_0$, which is represented by eq 5:

$$\Delta E = \gamma \hbar B_0$$  \[5\]

Electromagnetic radiation is produced when the excited nucleus transitions back to the lower energy state. The nuclear magnetic resonance (NMR) signal from a single proton is very weak so the signal detected in MRI is cumulative of all protons in the sample.
Figure 1. In the presence of an external magnetic field $B_0$, the magnetic moment $\mu$ will precess parallel to $B_0$ at the Larmor frequency, $\omega_L$.

Protons in this case follow a Boltzmann distribution shown in eq 6:

$$\frac{N_+}{N_-} = \exp\left[\frac{\gamma h B_0}{k_B T}\right]$$  \[6\]

where $N_+$ and $N_-$ refer to the lower and higher energy states of the spins, respectively. It is energetically favorable for protons to remain in the lower energy state ($N_+$) and thus a net vector magnetization $\vec{M}$ will be produced in the direction of the main magnetic field $B_0$. At equilibrium there will be no transverse component, i.e. there will be no magnetic vector in the $xy$ plane on an orthogonal coordinate system as shown in Figure 2.
Figure 2. B₁ field or rf pulse application. This figure shows the example case for a 90° rf pulse. Immediately after the pulse, net magnetization will be in the transverse plane and not in the longitudinal plane.

Considering eqs 4-6, when energy is applied to the magnetic vector in the form of a radio frequency (rf) pulse at the resonant frequency, protons are excited from the lower to higher energy state called resonance absorption. When the rf pulse is removed, the protons emit electromagnetic radiation at the same frequency as they return to the lower energy state.

According to classical theory, if the rf pulse $\vec{B}_1$ is applied perpendicular to the static magnetic field at the appropriate frequency, the rf pulse will couple to $\vec{M}$ causing $\vec{M}$ to rotate perpendicular to both $\vec{B}_0$ and $\vec{B}_1$. Figure 2 shows the effect of an rf pulse applied long enough to cause a 90° rotation of $\vec{M}$ such that the magnetization is completely in the transverse plane (3,4).
Relaxation

By applying an rf pulse $\vec{B}_1$ at the appropriate frequency, the macroscopic magnetization can be tipped out of the $z$-axis and, specifically for example a $90^\circ$ pulse, into the transverse plane. When the rf pulse is removed, relaxation will occur in which the protons will release the energy that they absorbed during the rf pulse. The final deposition of the energy produced from relaxation manifests itself in two ways; either to its surroundings in spin-lattice ($T_1$) relaxation or by spin-spin ($T_2$) relaxation. Relaxation is fundamental to the contrast in an MR image because different tissues, as well as diseased versus healthy tissue, have different relaxation characteristics allowing for the differentiation between adjacent tissues.

Spin-Lattice Relaxation

At equilibrium $M_z^0$ is parallel to the direction of $\vec{B}_0$. Energy absorption upon an rf pulse at the resonant frequency will cause $\vec{M}$ to rotate into the transverse plane. After the rf pulse is turned off, spin-lattice, or longitudinal ($T_1$), relaxation describes the process where the protons release their energy to the surroundings to once again align with $\vec{B}_0$. The $T_1$ relaxation time is defined as the time it takes for 63% of the magnetism to return to its original magnitude. For example, if a $90^\circ$ pulse is applied as shown schematically in Figure 3, then there will be no magnetization observed in the longitudinal plane immediately following the pulse. After time, however, magnetization will accumulate in its original direction according to eq 7:

$$M_z(\tau) = M_z^0 \left[ 1 - \exp\left(-\tau/T_1\right) \right]$$  [7]
where $\tau$ is the amount of time following an rf pulse. At a time approximately three times
the length of $T_1$, the magnitude of the magnetization in the $z$-direction, will be 95% of the
magnitude of $\tilde{M}$. Observing eq 7, it is apparent that the higher the longitudinal
relaxation rate ($1/T_1$) of a tissue, the faster magnetization will accumulate in the $z$-axis
following an rf pulse (4).

Spin-Spin Relaxation

Immediately after a $90^\circ$ rf pulse for example, magnetic coherence will be in the
transverse plane shown in Figure 4. Magnetization in the $xy$-plane will be highest upon
application of the rf pulse. Immediately after the rf pulse is removed, the protons will
undergo spin-spin, or $T_2$, relaxation. In $T_2$ relaxation energy is transferred to surrounding
protons that are also precessing near the Larmor frequency. Thus the energy remains as spin excitation unlike energy loss via $T_1$ mechanisms. The spins in the transverse plane start to dephase, resulting in an exponential loss of signal $\bar{M}$ in the $xy$ plane (eq 8) as shown in Figure 4:

$$M_{xy}(\tau) = M_{xymax} e^{-\tau/T_2}$$

where again $\tau$ is the amount of time following an rf pulse and $1/T_2$ is the relaxation rate of the voxel of interest. Inhomogeneities in the $B_0$-field also cause dephasing. Dephasing caused by these inhomogeneities is a consequence of the MR scanner and not reflective of the sample being imaged.
MR Image Generation

A fundamental requirement to produce MR images is to generate a position dependent magnetic field and to selectively excite desired protons by time varying magnetic gradients, which is accomplished by a gradient coil assembly residing inside the magnet core. A gradient coil assembly consists of three orthogonal gradient coils. An in depth analysis of gradient formation and slice selection is given elsewhere (3) and is beyond the scope of this manuscript. Briefly, if secondary gradients are applied perpendicular to the slice gradient, it is possible to manipulate the phase of the magnetization in the plane depending on the location in the slice and thus it is possible to reconstruct an image from NMR signal. NMR signal is collected successively from the sample in the Fourier (k-space) domain over time. Applying a discrete fast Fourier transformation (FFT) to the k-space matrix returns the resulting image, where the magnitude is dependent on the spin (proton) density and $T_1$ and $T_2$ relaxation rates of these protons.

Pulse sequences describe how the excitation rf and the gradients are turned on and off accordingly and repeated. Pulse sequences are required to obtain an image of a 2D slice or a 3D volume. Several types of pulse sequences exist, such as gradient echo, spin echo, and inversion recovery. Several parameters can be manipulated to obtain optimal contrasts. One parameter that all pulse sequences have in common is the repetition time ($TR$), or the time between successive excitation pulses for a given slice. Spin echo and gradient echo pulse sequences also depend on the echo time ($TE$) of the protons in the transverse plane. In spin echo type pulse sequences the length of $TE$ determines the amount of $T_2$ weighting. Eq 9 describes the signal from a spin-echo pulse sequence.
In this example, by manipulating imaging parameters, such as TR and TE, it is possible to weight the image based on the characteristics of the desired protons. If TR is long and TE is short, the image will be characteristic of the proton densities in the slice. If both TR and TE are long, the image will be T2-weighted, where protons that have short T2 relaxation times will appear darker. In a T1-weighted spin echo image, both the TR and TE are short so that protons with higher longitudinal relaxation rates have higher signal. This is just one example of an imaging pulse sequence that demonstrates how MR images can be manipulated to achieve tissue enhancement based on variable physiology \((1,3,4)\). Other pulse sequences in this manuscript are explained where necessary.

Contrast Enhanced MRI

Although MR images can obtain contrast based on manipulation of the imaging parameters alone (see above), contrast in an MR image needs to be optimized to give the best possible contrast between tissues. Additional contrast enhancement requires the use of a paramagnetic contrast agent. Specifically, the aim of MR contrast agents is to decrease T1 and/or T2 relaxation times of water protons proximal to the contrast agent by dipolar interactions. Depending on whether a T2 or T1 enhancing contrast agent is used, the signal of water proximal to the contrast agent will be decreased or increased, respectively, compared to water protons in surrounding tissues.

Paramagnetic materials based on gadolinium(III) [Gd(III)], manganese(II), and iron oxide particulates are often used to achieve proton relaxation \((5,6)\). These agents owe their paramagnetism to one or more unpaired electrons and therefore have an electronic
paramagnetic moment. The vast majority of clinical contrast enhanced exams use Gd(III)-based contrast agents; thus the focus here will be contrast agents developed using Gd(III) ions. Gd(III) ions are characterized by seven unpaired electrons leading to a very large magnetic moment (5). In addition, Gd(III) has a symmetric S-state, which means it has a much slower electronic spin relaxation rate that can easily interact with the frequency of a proton. Compared to other lanthanide metals, such as dysprosium(III) and holmium(III), Gd(III) has a lower magnetic moment. However, Dy(III) and Ho(III) have asymmetric electronic states leading to high electron spin relaxation to the extent that these metals have almost no interaction with proximal protons. Therefore, Gd(III) is the optimal metal for enhancing the relaxation rate of protons (7).

Gd(III) interacts both directly and indirectly with surrounding water molecules. Specifically, for clinically approved Gd(III) complexes water binds in the first coordination sphere of the metal ion and becomes magnetically altered. This process is called inner-sphere relaxation. This water molecule then exchanges into the bulk water and affects their magnetism via dipole-dipole interactions as shown schematically in Figure 5 (8). Several factors govern the inner-sphere proton relaxivity, such as the hydration number, Gd-H distance, water exchange rate from the inner sphere to bulk water, the rotational properties of the molecule, and electron relaxation. Water that diffuses into the area around the Gd(III) ion can also be susceptible to its paramagnetic effect, which is referred to as outer-sphere relaxation. Although both types of relaxation contribute to the observed relaxivity of water, the factors controlling inner-sphere relaxation can be manipulated since direct binding of a water molecule to Gd(III) is required (8).
Figure 5.  Gd(III)-water exchange. For clinically approved contrast agents, the water exchange mechanism occurs most significantly with the inner coordination sphere.

The chemical nature of the Gd(III) chelate and its effect on relaxivity is an important consideration in the design of a contrast agent. The observed solvent relaxation rate is composed of both the diamagnetic and paramagnetic relaxation rates (eq 10).

\[
\frac{1}{T_{i,\text{obs}}} = \frac{1}{T_{i,d}} + \frac{1}{T_{i,p}}, \text{ where } i = 1, 2
\]  

[10]

The diamagnetic term, \(1/T_{i,d}\), is the relaxivity contribution of the native solvent with no paramagnetic compound present. The second term, \(1/T_{i,p}\), represents the enhancement in relaxivity due to the presence of a paramagnetic compound. Considering Gd(III) in dilute
solution, the paramagnetic term is linearly proportional to its concentration as shown in eq 11 (7).

\[
\frac{1}{T_{i,\text{obs}}} = \frac{1}{T_{i,d}} + r_i[Gd^{3+}], \quad \text{where } i = 1, 2
\]  \hspace{1cm} [11]

Thereby, plotting the observed relaxation rates for various concentrations of Gd(III) yields a linear fit in which the slope characterizes the relaxivity, \( r_i \) (typically units of mM\(^{-1}\)s\(^{-1}\)) of the molecule. Proton relaxivity is the efficiency of a paramagnetic substance to enhance the relaxation rate of water protons, and therefore is reflective of its efficacy as a contrast agent. In the case of \( T_1 \) contrast agents, this results in increased signal enhancement in the solvent proximal to Gd(III). Consequently, if a contrast agent has a high relaxivity, it is a more effective contrast agent (8).

Gadolinium(III) Complexes as Contrast Agents

Gd(III) ions cannot be administered directly because they are highly toxic in their ionic form. Gd(III) ions are potent inhibitors of Ca\(^{2+}\) channels in endocrine cells (9,10) and can influence Ca\(^{2+}\) binding sites of proteins and enzymes (11-14). Rats administered GdCl\(_3\) intravenously showed signs of acute toxicity, including decreased respiration, lethargy, abdominal cramps, diarrhea, and complete cardiovascular collapse, with a low LD\(_{50}\) of 0.5 mmol-Gd/kg (15,16). Acute neural toxicity after intracisternal administration of gadolinium chloride, measured by the ED\(_{50}\), the dose at which 50% of the tested population loses motor coordination, was 6 mmol-Gd/kg (16). Another major toxicity concern of Gd(III) is the long-term bone accumulation. It was shown in rats that 40% of the injected dose still remained in the skeleton 8 months postinjection of gadolinium.
citrate (17). In addition, after 12 weeks, rats injected with gadolinium chloride showed perinuclear vacuolization of the parenchymal cells of the liver and course granularity of the liver (17).

By chelating Gd(III) to a ligand, the toxicity is greatly reduced. There are two main ligand structures on which clinically approved contrast agents are based; a linear ligand, diethylenetriaminepentaacetic acid (DTPA), and a macrocyclic ligand, 1,4,7,10-tetra(carboxymethyl)-1,4,7,10-tetraazaacyclododecane (DOTA). For example, gadolinium dimeglumine, the first MRI contrast agent approved for clinical use, has a reported LD50 of 5.6-10 mmol/kg in mice compared to 0.5 mmol/kg for GdCl3 (16,18). In general clinically approved contrast agents based on Gd(III) are characterized by low molecular weight, stable complexation of Gd(III) to DTPA or DOTA chelating ligand (and their derivatives), relatively low relaxivity, and extravascular and extracellular distribution with rapid elimination. Figure 6 shows the chemical structure of MRI contrast agents that are clinically approved in the United States.

Since the FDA first approved [Gd(DPTA)(H2O)]2− in 1988 for adult CNS indications, paramagnetic Gd(III) complexes have become an essential tool in the detection and characterization of many diseases. These low molecular weight Gd(III) complexes are most effective in diagnosing diseases of the CNS where there is a breakdown of the blood-brain barrier (19-21) representing neurodegenerative disease such as multiple sclerosis (22) where the small contrast agents can permeate into the usually inaccessible tissue. These contrast agents have also shown utility in diagnosing and characterizing diseases of the cardiovascular system. With the potential of angiographic MR techniques, vascular imaging of the coronary (23) and cerebral arteries
Figure 6. Clinically approved MRI contrast agents. Gd(III)-based MRI contrast agents that were clinically approved in the United States as of June 2006. Coordination bonds and H$_2$O have been left out for clarity.

(24), pulmonary (25) and peripheral vessels (26), and renal artery stenosis (27) shows promise.

More recently, MRI contrast agents that are tissue and organ selective, especially for hepatocytes, are becoming clinically available (28). Gd-BOPTA was approved by the FDA for clinical use in 2004. This agent has a slightly hydrophobic side chain, which promotes liver targeting and bilary excretion. Gd-BOPTA is indicated and relatively effective at detecting focal liver lesions in patients with known or suspected primary liver
cancers (29,30). There are also a number physiochemically and bioactive MRI contrast agents under development for specific applications, such as PARACEST and pH sensitive contrast agents (31-35).

The physiochemical properties of clinically approved low molecular weight agents have been well characterized and are reviewed elsewhere (7,8). In short, the molecular weights of all the contrast agents in Figure 6 are less than 700 Da (based on exact molecular weight of Gd(III) and the ligand). The relaxivity for these agents is similar, but relatively low, ranging from 3.7 mM⁻¹ s⁻¹ for Gd(HP-DO3A) to 5.2 mM⁻¹ s⁻¹ for Gd-BOPTA (7). Chemical modification yielding novel chelates has been performed to improve relaxivity (34,35), but none of these agents are clinically approved.

The high metal to chelate stability of these agents ranges from 16.9 to 23.8 M⁻¹ (8) resulting in extremely low (< 0.1% ID) to undetectable bone uptake (36-38). The LD₅₀ dose in mice for ionic agents Gd-DTPA and Gd-BOPTA is 5.6 and 5.8 mmol-Gd/kg, respectively (18,39). Nonionic compounds, Gd(DTPA-BMA) and Gd(HP-DO3A), have an LD₅₀ of 14.8 and 12 mmol-Gd/kg, respectively, likely due to lower osmolality (18,40). In general, these agents improve contrast enhancement compared to MR images that are not contrast enhanced by a paramagnetic compound. Most importantly, these agents also have acceptable tolerance profiles.

**Limitations of Low Molecular Weight Gd(III) Complexes**

At clinical doses, clinically approved MRI contrast agents are characterized by rapid elimination. The terminal phase half-life of these agents is quite short at 10-20 min in rats and less than 1 h in humans (41,42). In addition, the contrast agents are classified as extracellular (7), meaning that the blood pool concentration of these agents decreases
rapidly after injection from both elimination and extravasation out of the vasculature. Since low molecular weight Gd(III) chelates can rapidly diffuse across healthy vascular endothelium, $T_1$ shortening of surrounding tissue decreases the overall image quality for angiographic images\(^{(41,43)}\). In addition, the rapid blood pool clearance results in difficulty of exact timing between the contrast agent bolus and image acquisition\(^{(44)}\). As a result, the potential image resolution is greatly compromised, especially in MR protocols where imaging of the blood pool is desired, such as cardiovascular disease and potentially cancer imaging.

**Macromolecular (Blood Pool) MRI Contrast Agents**

To overcome the limitations of low molecular weight contrast agents, such as rapid clearance from the blood pool, nonspecific extravasation into surrounding tissue, and subsequently suboptimal performance at reducing $T_1$ relaxation times in blood, attention shifted to the development of macromolecular Gd(III) complexes for contrast enhanced MRI. The development of macromolecules as therapeutic carriers or agents in drug delivery is well recognized. These same attributes, such as decreased elimination and confinement to the blood pool also make macromolecules an ideal choice for angiographic imaging. Unlike clinically approved low molecular weight contrast agents, macromolecules do not readily diffuse across healthy vascular endothelium. In fact, macromolecules tend to diffuse across only endothelium that has been compromised and preferentially accumulate via the enhanced permeability and retention (EPR) effect\(^{(45,46)}\). This will occur wherever an inflammatory response is present, such as cancer, vascular disease, and arthritis. In the case of diagnostic imaging this gives desired preferential enhancement in the diseased tissue. In addition, macromolecular Gd(III)
complexes typically have a higher relaxivity than low molecular weight complexes, which means that macromolecules are more efficient contrast enhancers (41). Combined with the fact that the distribution of macromolecules is preferential, lower doses of Gd(III) are possible with macromolecular Gd(III) complexes. These qualities make macromolecules the likely choice to overcome the limitations of currently available Gd(III) complexes for MRI for more effective MR imaging. The following section will outline the design and development, physiochemical properties, and in vivo properties of the general classes of blood pool MRI contrast agents.

**Synthetic Macromolecular Blood Pool Contrast Agents**

Synthetic macromolecular contrast agents for blood pool MR imaging first appeared in the literature in 1990 (47-49) and are an active area of research to date. Synthetic macromolecules for blood pool MR imaging include polyamino acid backbones conjugated to Gd(III) chelates, copolymers of Gd-DTPA and a diamine or a dialcohol, and dendrimeric (cascade) polymer platforms. Due to the synthetic nature of these macromolecules a wide variety of structures have been developed to fit specific MR applications.

Polyamino acids, especially polylysine, are routinely tested as therapeutic carriers for drug delivery because of their positive charge at physiological pH, which allows for noncovalent complexation to negatively charged therapeutic molecules, such as DNA. Covalent conjugation of a therapeutic moiety with the ε-amino groups of polylysine can also be utilized for drug delivery. With respect to developing macromolecular MRI contrast agents, either Gd-DTPA or Gd-DOTA have been covalently linked to the polylysine backbone with a direct covalent bond of the carboxylate group of DTPA (50)
or DOTA (47) or via linking groups (32,51). The $T_1$-relaxivity of these contrast agents was reported to be 10.9 to 13.6 mM$^{-1}$s$^{-1}$ (49,50,53), much greater than that of clinically approved low molecular weight agents, possibly due to a slower rotational correlation coefficient. Polylysine based macromolecular contrast agents have been synthesized in a wide range of molecular weights with varying pharmacokinetics. PLL-GdDTPA of 36 kDa had a half-life in blood of 64.9 min, while PLL-GdDTPA of 480 kDa had a half-life of 428.6 min in rats (53). At 7 days postinjection of PLL-GdDTPA (48 kDa), greater than 3% of the injected dose resided in the liver, kidney, and bone of rats (50). In general, PLL based macromolecular contrast agents have relatively low immunogenicity (54), but show poor hemodynamic tolerance at higher doses (55).

The diagnostic applications of PLL-GdDTPA have been extensively investigated in vivo. High molecular weight PLL-GdDTPA (407 kDa) showed higher and more sustained contrast enhancement in a mammary adenocarcinoma rodent model (56). PLL-GdDTPA (40 kDa, 40 GdDTPA) was able to accurately differentiate between two causes of pulmonary edema; hydrostatic pressure and abnormal capillary permeability (57), and in another case detected a pulmonary embolism (58). Regarding myocardial infarctions, differential enhancement patterns of PLL-GdDTPA were effective in delineating the central and peripheral ischemic zones in cats (59) and differentiating acute, subacute, and chronic myocardial infarctions in rats (60).

A second class of synthetic macromolecular contrast agents is the copolymers of the Gd-chelating ligand and an $\alpha,\omega$-diamine or -dialcohol, such as diamino-poly(ethylene glycol), diamino-poly(propylene glycol), or diamino hexane for example (52,61-63). The molecular weight of copolymers from GdDTPA and either PEG or PPG, ranges from
10 kDa to 83 kDa with relaxivity values ranging between 3.8 and 6.0 mM\(^{-1}\)s\(^{-1}\) for PEG and PPG based copolymers with DTPA (61,63). When an alkylidiamide is considered, the relaxivity increases with more methylene groups; values up to 20 mM\(^{-1}\)s\(^{-1}\) have been reported, suggesting that hydrophobic interaction may limit molecular motion around Gd(III) (62), whereas free rotation occurs around PEG-DTPA copolymers (64).

One of the furthest developed contrast agents from this class, poly-[Gd-DTPA]-co-[1,6-diaminohexane] (19 kDa) (Nycomed Inc., Wayne, PA), has a \(T_1\) relaxivity of 9.5 mM\(^{-1}\)s\(^{-1}\) at 20 MHz (65). In vivo, this contrast agent is characterized by a 98 min half-life in rabbits with 4.91% ID remaining in bone after 7 days (52). In an arterial stenosis model in rabbits, the extended half-life of the agent produced stable enhancement for 2D and 3D TOF-MRA images (66). In other studies, a subcutaneous injection poly[Gd-DTPA]-co-[1,6-diaminohexane], showed excellent popliteal nodal enhancement in a rabbit model. In mice with a human melanoma xenograft, dynamic enhancement of the copolymer was used to predict the efficacy of a macromolecular therapeutic (67).

The third major group of synthetic macromolecular blood pool agents is dendrimer-based complexes. Dendrimers are highly branched polymers consisting of three fundamental components: a core, the branching units, and a layer of functional groups to be modified depending on the application (68). There are several advantages of dendrimers for diagnostic application including uniform molecular weight distribution, relatively controlled structure compared to linear polymers, increased molecular weight compared to clinically approved agents, and typically higher relaxivity because dendrimers have a comparatively more rigid structure than linear polymers (69). The first published reports of dendrimers for MRI applications were in 1994 by Adam et al.
where they were called cascade polymers, and Wiener et al. (71). Typical core types include, but are not limited to ammonia, ethylenediamine, diaminobutane, and trimesoyl triamide. Branching units also have a wide variety of chemical structures, such as polyamidoamine (PAMAM), polypropyleneimine, and lysine. Either GdDTPA or GdDOTA can be conjugated to the terminal amines. Detailed reviews are given by several groups on the cores, structures, physiochemical properties, pharmacokinetics, and various MRI applications of paramagnetic dendrimer complexes (68,72,73).

In brief, PAMAM-based dendrimeric contrast agents range in molecular weight from 15 kDa (generation 2, 16 Gd ions) to 3820 kDa (generation 10, 4096 Gd ions) with relaxivity values from 20 (generation 2) to 35 mM⁻¹s⁻¹ (generation 8, 954 kDa) (73,74-77). The dynamic enhancement pattern of PAMAM-based dendrimeric contrast agents depended significantly on the generation number, with generation 4-6 providing the best signal enhancement of small vessels (74), while an ethylene diamine core provided more efficient signal enhancement than an ammonia core (78). A comparison of different branching units, PAMAM versus polypropyleneimine diaminobutyl (DAB, \( r_1 = 12 \) to 29 mM⁻¹s⁻¹), showed that DAB-based dendrimers had lower whole body accumulation compared to PAMAM, but significantly higher liver accumulation (75). The selective accumulation of DAB dendrimers has led to their preclinical development as effective liver specific imaging agents (79,80). PAMAM dendrimers, on the other hand, were effective in identifying tumor vasculature (81,82) and predicting herceptin internalization (83). Both DAB and PAMAM dendrimers have shown efficacy in visualizing the lymphatic system in mice (76,84).
An agent in the dendrimeric group of contrast agents, which is in clinical development, is Gadomer-17 (Shering AG, Berlin, Germany). Gadomer-17 has a molecular weight of 17.5 kDa and has a relaxivity of 16.5 mM$^{-1}$s$^{-1}$ at 20 MHz. This dendrimer has a trimesoyl triamide core, 18 lysine residues to bind 24 GdDOTA molecules (69). The molecule is large enough that its extravasation out of the blood pool is reduced, but the molecule is still small enough that it is cleared via glomerular filtration as characterized by a terminal phase half-life of 10 min in rabbits (85). This contrast agent has been tested with some success in several types of imaging, especially angiography. It has also been used to characterize tumor severity and angiogenesis (86).

Natural Macromolecule-Derived Blood Pool Contrast Agents

Several macromolecular Gd(III)-based MRI contrast agents have been developed from natural biomacromolecule platforms, such as protein conjugates, polysaccharides, and dextrans. The first such agents consisted of DTPA covalently linked to the amine groups of proteins, such as native albumin (87), IgG (88), fibrinogen (89), and inulin (90,91). Of these, the most comprehensively researched biomacromolecule is albumin. On average, the synthesis yields 19 to 35 DTPA molecules to albumin (87,92), resulting in a contrast agent with an apparent molecular weight of 92 kDa. After Gd(III) complexation, the relaxivity of the agent is 14.9 mM$^{-1}$s$^{-1}$ at 0.25 T, significantly higher than clinically approved agents. The pharmacokinetics of this agent reveal that it is limited to the intravascular space, but due to extremely slow elimination (93), Gd(III) dissociation (88) and potential immunogenicity (54), it has been developed only as a prototype agent. Nevertheless, albumin-[GdDTPA]$_x$ has made a large contribution to understanding macromolecular contrast agent dynamics with regard to noninvasive tumor
characterization and has helped to expose the potential clinical utility of a macromolecular contrast agent (92,94-99).

Blood pool macromolecular contrast agents have been derived from other natural macromolecules in addition to proteins, such as polysaccharides and their derivatives. For example, both DTPA and DOTA have been conjugated to polysaccharides (100), while DTPA has also been used as a cross-linking agent between polysaccharides (101). The molecular weight of cross-linked polysaccharides ranged from 17-150 kDa, while the relaxivity of these compounds ranged from 5.3 to 6.5 mM⁻¹s⁻¹ (101). Carboxymethyl dextran-GdDTPA (CMD-GdDTPA) ranged in molecular weight from 42.7 to 158 kDa with relaxivity values from 6.5 to 7.3 mM⁻¹s⁻¹ (100,102,104). With DOTA as the chelating ligand (CMD-GdDOTA), contrast agents were approximately 50 kDa and had a longitudinal relaxivity of 10.6 mM⁻¹s⁻¹ (105). Both CMD-GdDTPA and -GdDOTA showed better enhancement than a low molecular weight control for MR investigations involving angiography, especially those relating to myocardium (102,105-107), and assessing liver disease (103,108).

**Colloidal Blood Pool Contrast Agents**

Blood pool contrast agents have been developed from colloidal molecules, such as liposomes and micelles. With respect to liposomes, Gd(III) complexes can either be encapsulated in the aqueous environment of the liposomal cavity or Gd(III) can be complexed at the polar end of amphiphilic DTPA derivatives. Liposomes encapsulating GdDTPA have been prepared from egg phosphatidylcholine/cholesterol, dioleoylphosphatidylcholine/cholesterol, and dipalmitoylphosphatidylcholine/cholesterol. The size of the particles range from 20 – 400 nm for unilamellar vesicles (109,110) and
>10 microns for large multivesicular liposomes (111). Unlike other proposed blood pool agents, liposomes possess a relatively low $T_1$ relaxivity, dramatically lower than the relaxivity from GdDTPA itself. Unilamellar vesicle encapsulated GdDTPA had a $T_1$ relaxivity of 0.42 to 3.43 mM$^{-1}$s$^{-1}$ (109,112), where the relaxivity decreased with increasing particle size (109). These findings were attributed to poor water flux across the lipid bilayer. Although liposome encapsulated Gd(III) complexes have shown some utility for imaging of the reticuloendothelial system organs and the brain (111,113), the development of these particles for MR imaging has been severely limited due to toxicity concerns. The acute toxicity of these agents is similar to Gd-DTPA (110). However, these agents resulted in significant deposition of Gd(III) (13 days) in the spleen, liver, and lung (112,114). Signs of toxicity included significant splenomegaly, lymphocytopenia, and hypergammaglobulinemia. Damage to cells of the spleen was the most significant of all the observed toxicities (115).

Amphiphilic GdDTPA derivatives, stearyl ester (GdDTPA-SE), stearyl thiolester (GdDTPA-ST), stearyl amide (GdDTPA-SA), and decylamide (GdDTPA-DA), have been investigated to increase the relaxivity of liposomal GdDTPA by making the paramagnetic metal readily accessible to water (116-118). When GdDTPA was part of the bilayer, some increase in $T_1$ relaxivity was observed, but like liposomally entrapped GdDTPA, these agents also showed long-term Gd(III) accumulation in the RES. At 12 days, GdDTPA-SA liposomes had 62% Gd(III) retention in the liver and 10% in the spleen (118). GdDTPA-SE and GdDTPA-ST resulted in 30% and 8% ID, respectively, in the liver after 10 days, while GdDTPA-DA showed 2% (116,118). In the spleen, the accumulation from GdDTPA-SE and GdDTPA-SA was 6% and 5% of the injected dose,
respectively, remaining after 10 days. Nearly constant values were obtained from all
time points measured over that interval, showing that this accumulation was indefinite
(118).

Micelles have also been investigated for MRI applications. GdDOTA was
conjugated to alkyl chains that differed in the number of carbons on the chain (119). The
relaxivity of the micelles were 10.8 to 22.0 mM\(^{-1}\)s\(^{-1}\), where the shortest alkyl chain had
the lowest relaxivity. Mixed micelle formulations showed good vascular enhancement in
an MRA protocol (120). A Gd(III) retention experiment of the mixed micelle showed
that 8% to 10% of the injected dose still remained after 7 days and 5% remained after 28
days.

Small Molecule Protein-Binding Blood Pool Contrast Agent

This class of compounds is not comprised of high molecular weight agents, but of
small molecules (MW < 1200 Da) with a hydrophilic Gd(III) complex and a hydrophobic
region for reversible, noncovalent binding to serum albumin (121). In their unbound
form, these contrast agents have relatively low relaxivity, but when bound to albumin, the
\(T_1\) relaxivity increases from 25 to 35 mM\(^{-1}\)s\(^{-1}\). In addition, when bound to the protein, the
contrast agent exhibits the properties characteristic of a macromolecular contrast agent,
such as a long terminal half-life and confinement to the vascular pool.

The most well-studied contrast agent of this class is MS-325 (122). This agent has a
pentaacetic acid derivative for Gd(III) complexation, linked via a phosphodiester bond to
a hydrophobic diphenylethoxycyclohexyl moiety for albumin binding. The binding of this
molecule to albumin and its effect on relaxivity has been well studied. At physiological
conditions, approximately 88% of MS-325 is bound to albumin with a binding constant
of up to 11.0 mM$^{-1}$ (123). The Gd(III) complex formed with MS-325 was determined to be more stable than a clinically approved agent and significantly more chemically inert (124). In preclinical and clinical experiences, MS-325 has shown application for angiographic experiments (125-128). However, when MS-325 was applied to a chemically induced rat tumor, it was not as effective as a prototype macromolecular contrast agent (GdDTPA-albumin) at identifying tumor grade (129).

A second contrast agent from this group, gadocoletic acid trisodium salt, has also shown potential. This contrast agent uses an analog of deoxycholic acid for albumin binding. The relaxivity of gadocoletic acid increases from 6.4 mM$^{-1}$s$^{-1}$ to 27 mM$^{-1}$s$^{-1}$ upon binding to albumin (130). In human plasma, 94.5% was bound to albumin, slightly higher than the percent bound under similar condition from MS-325 (131). Gadocoletic acid has shown efficacy in coronary angiography (132).

**Limitations of Macromolecular Gd(III) Complexes**

Safety is the first priority in the development of any contrast agent for MRI. Clinically approved low molecular weight contrast agents are all rapidly eliminated by, for the most part, renal filtration. Therefore, potential long-term toxicity from Gd(III) accumulation is minimal. The rapid elimination from the vasculature by low molecular weight agents results in poor performance in blood pool imaging applications, however. For more effective MRI, improvements are necessary to increase circulation time.

Macromolecular MRI contrast agents have definitive advantages compared to low molecular weight agents as shown above. The major reason for their efficacy is that their size limits renal filtration, normally when the molecule is greater 20 kDa. Above 20 kDa renal filtration depends on several physiochemical characteristics, such as molecule
lipophilicity and polarity (133). Potentially, agents whose molecular weight is greater than 20 kDa and very large macromolecules (>70 kDa) must be metabolized before being eliminated (41). This increases the potential for cellular uptake of the contrast agent via endocytosis and consequential Gd(III) release may occur due to the decreased pH in the lysosome (134).

Gd(III) accumulation has in fact limited the clinical development of macromolecular MRI contrast agents. For example, the linear copolymer of GdDTPA and diamino hexane (19 kDa) had 4.91% ID in the bone at 7 days postinjection, which was 23 times higher than a control agent GdDTPA (52). PLL-GdDTPA (42 kDa) had more than 3% ID in each the liver, kidney, and bone at 7 days (50). A second-generation polypropyleneimine dendrimer (7 kDa) resulted in 45% ID retention 14 days after injection (135). A nine-generation dendrimer reported almost 60% ID 4 days postinjection (136). Carboxymethyl hydroxyethyl starch-(Gd-D03A)35 (72 kDa) resulted in 47% of the injected dose remaining in the body after seven days (137). As stated above, the toxicity from liposomally entrapped GdDTPA is prohibitively high. In addition to concerns from long-term Gd(III) accumulation, the chemical structure of the contrast agent itself may produce a toxic response. Several macromolecular contrast agents investigated have potential immunogenic responses (54), while incomplete ligand coupling to primary amines in the backbone of dendrimeric agents (77) may result in poor hemodynamic tolerance and accumulation of contrast agents in kidney.

In light of the potential toxicity from macromolecular Gd(III) complexes, several alternative approaches have been proposed to balance prolonged circulation and long-term Gd(III) deposition. Contrast agents have been synthesized below the renal
threshold, but that are larger than clinically approved agents; examples include, Gadomer-17 (138), P760 (139), and P792 (140). These so-called, rapid clearance blood pool agents potentially work better than clinically approved low molecular weight contrast agents, but they did not give definitive data in a tumor model compared to larger macromolecules (141,142). To date, none of these contrast agents have been clinically approved.

Chemical modification to existing macromolecular Gd(III) complexes has also been reported in the literature. Poly(ethylene glycol) has been grafted to GdDTPA-PLL to help improve biocompatibility (143). Dendrimeric contrast agents have also been PEGylated resulting in decreased liver accumulation and increased renal excretion (144). Dendrimeric contrast agents have also been coadministered with lysine (145) or biotinylated (146) to facilitate renal excretion. Recently, PEGylated liposomes were prepared and showed distribution to vasculature (147), but long-term Gd(III) retention studies with these agents have not been reported.

**Statement of the Problem**

Nearly one-third of MRI exams are currently contrast enhanced by low molecular weight Gd(III) complexes (7). These contrast agents aid in the diagnosis of a wide range of pathologies by enhancing the morphology and functionality of the tissue (28,32,86,92,148). These agents have little inherent toxicity because their small size allows them to be readily eliminated, mostly via renal filtration (37,38,42). In addition, DTPA and DOTA ligands and their derivatives form a stable complex with Gd(III) (7). Unfortunately, the rapid elimination that renders these agents safe for clinical use also results in undesirable pharmacokinetic performance, including inadequate blood pool
retention time for vascular imaging applications. These agents are also extravascular meaning they diffuse from the vasculature through healthy endothelium in addition to endothelium of diseased tissue, which decreases the relative enhancement between the tissue of interest and surrounding tissue (149). Finally, these agents are inefficient at shortening $T_1$ values, forcing a higher Gd(III) dose to achieve the same $T_1$ shortening as an agent with higher relaxivity (8). The major consequence is that these agents have suboptimal imaging performance when blood pool retention is desired, such as for cardiovascular and tumor imaging (41,150).

Macromolecular Gd(III) complexes are an effective alternative to extravascular agents because the large size of macromolecules results in a long circulation half-life, confinement to the blood pool, and increased signal intensity, all of which allow for a more complete data acquisition from the MR exam. Macromolecular contrast agents have been developed by the conjugation of Gd-DOTA, Gd-DTPA, or their derivatives to synthetic polymers, such as polylysine and dendrimers (47-86), biological macromolecules (87-108), or incorporated into colloidal particles (109-120). In preclinical studies, their utility for assessing diseases of the cardiovascular system, tumors, and other tissues has been clearly demonstrated (56-60,66,67,76,79,86,92,94-99,102,105,107,108,125-128,132). Unfortunately, the clinical development of these agents is limited because their slow clearance may result in a metabolic release of toxic Gd(III) ions (135-137), while other macromolecular contrast agents are potentially immunogenic (50,52,54). The key point is that no matter how efficient a macromolecular Gd(III) complex is in preclinical studies, it will not be of clinical benefit unless it demonstrates safety comparable to clinically approved MRI contrast agents. Until this
condition is met, the potential of a macromolecular MRI contrast agent cannot be realized. The content of this dissertation presents the design and development of a novel approach to achieve the maximum potential of a blood pool contrast agent combined with the safety characteristics of a low molecular weight Gd(III) chelate.

**Literature Cited**


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CHAPTER 2

PROJECT RATIONALE

To address the challenge of designing a contrast agent with the imaging attributes of a macromolecular MRI contrast agent, while achieving the favorable Gd(III) clearance profile of a low molecular weight chelate, novel biodegradable macromolecular contrast agents have been developed. These contrast agents have disulfide bonds incorporated into a polymeric backbone. Potentially, these disulfide bonds can be readily reduced by the thiol-disulfide exchange reaction with endogenous or exogenous thiols facilitating the clearance of low molecular weight Gd(III) chelates. This chapter underlines the rationale and presents the approach undertaken to achieve this goal.

The Disulfide Bond

The disulfide bond has a unique biological role, including, but not limited to protein folding and stabilization (1,2), involvement in the regulation of gene expression (3,4), and catalytic activity (5). Under milder redox conditions, such as in plasma compared to intracellularly, the disulfide bond is quite stable. In the presence of a free thiol, the disulfide bond can be readily cleaved via the thiol-disulfide exchange reaction (Figure 7). Mostly, these harsher redox conditions are located intracellularly due to a high concentration of reduced glutathione (GSH), approximately 10 mM. In contrast, the relatively lower redox potential of the plasma can be attributed to a total free thiol concentration of around 15 μM (6,7).
The considerable difference between the redox potential of disulfide bonds intracellularly and extracellularly has been extensively explored for drug delivery systems and is reviewed elsewhere (8). In most cases, the developed drug delivery system takes advantage of the milder reducing conditions of the plasma, which promotes a minimal plasma degradation of the drug delivery system. Upon cellular uptake of the system, the high intracellular thiol concentration is exploited to cause release of their cargo by the reduction of the disulfide bond. For example, drugs have been covalently linked to poly(D-lysine) (9) or antibodies and other proteins (10-12) via a disulfide containing linker. Alternatively, other researchers developed delivery systems in which either a container or hydrogel is held together by disulfide bonds (8,13). In these cases when the disulfide is reduced the hydrogel swells releasing the drug, or the container breaks away releasing the active drug compound.

The low plasma concentration of free thiols and consequently the relatively weaker redox potential in plasma is exploited in the current project. The toxicity associated with currently developed macromolecular contrast agents is resultant from prolonged circulation well after a reasonable time for the MRI exam (14-21) where Gd(III) ions are metabolically released upon intracellular uptake by the low pH of the endosome (22).
The ideal angiographic MR contrast agent would provide the positive attributes of a macromolecule, such as being limited to the intravascular space and increased circulation time, but after an amount of time sufficient for the MRI exam, the contrast agent could break down and be readily cleared. This presents the ideal circumstance to take advantage of the relatively lower redox potential of the disulfide bond in plasma. Incorporation of the disulfide bond into the macromolecular contrast agent would provide the advantages of a macromolecule, however, the disulfide containing macromolecule could gradually degrade because of the relatively low thiol concentration in the bloodstream and Gd(III) could be cleared from the bloodstream as shown schematically for the ideal case in Figure 8.

**During MR Examination**

Endothelial membrane

Glomerular membrane

**After MR Examination**

Endothelial membrane

Glomerular membrane

<table>
<thead>
<tr>
<th>polydisulfide Gd(III) contrast agent</th>
<th>S-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>monomeric Gd(III) complex</td>
<td>SH</td>
</tr>
</tbody>
</table>

Figure 8. Proposed distribution of polydisulfide Gd(III) complexes. Initially the polydisulfide Gd(III) complexes would be restricted to the blood pool with sufficiently long circulation half-life. In an ideal case, after the MR examination, the polydisulfide would be reduced by plasma thiols, which would facilitate the clearance of Gd(III) as small chelates.
Biodegradable Polydisulfide Gd(III) Complexes

Recently, contrast agents based on polydisulfides have been developed to facilitate the clearance of low molecular weight Gd(III) complexes (23). The first such agent developed was GdDTPA cystamine copolymers (GDCC), which is shown in Figure 9 (24). This contrast agent was synthesized by the copolymerization of cystamine and DTPA dianhydride in DMSO using TEA as the base, followed by Gd(III) complexation, and ranged in molecular weight from 15 to 60 kDa. The relaxivity of the complexes ranged from 4.4 to 6.3 mM⁻¹s⁻¹.

Verification of biodegradability via the thiol-disulfide exchange reaction was preliminarily investigated in vitro by the incubation of copolymer with L-cysteine. Theoretically, the free thiol of L-cysteine should exchange with the disulfide bond of L-cystine in the copolymer thus breaking the polymer into smaller units as shown in Figure 10. Size exclusion chromatography (SEC) results showed that as early as 5 min after incubation with 15 µM L-cystine there was a shift from high molecular weight to low molecular weight. The expected degradation products were also observed by MALDI-TOF mass spectroscopy of the reaction mixture. Incubation with HSA showed no evidence of a cross reaction with the Cys-34 residue even after 6 h of incubation of the copolymers with HSA (24).

![Chemical structure of GdDTPA-cystamine copolymers (GDCC).](GdDTPA)-Cystamine

Figure 9. Chemical structure of GdDTPA-cystamine copolymers (GDCC).
Biodegradable polydisulfide Gd(III) complexes also demonstrated potential blood pool contrast enhanced MRI application. GDCC produced more prominent contrast enhancement in the vasculature than a low molecular weight control, Gd(DTPA-BMA). The relative amount of enhancement was dose- and molecular weight- dependent. The biodegradable macromolecular agents excrete relatively quickly via renal filtration and showed long-term Gd(III) accumulation comparable to that of a control agent (12). Low molecular weight and oligomeric degradation products were identified in the mass spectra of urine samples collected from the rats injected with the agents (11, 13).
These preliminary studies with GDCC have highlighted the potential for biodegradable macromolecular MRI contrast agents. These agents can be readily synthesized to obtain a range of molecular weights. The contrast agents are readily degraded via the thiol-disulfide exchange reaction resulting in renal excretion as confirmed by MALDI-TOF MS. The contrast enhancement of these agents was more significant and was more persistent than that observed with a low molecular weight control. More sustained contrast enhancement would be desirable for longer imaging protocols. It would then be advantageous to develop a biodegradable macromolecular contrast agent based on polydisulfides with tunable pharmacokinetics. One potential method to achieve this goal is to structurally modify the chemistry around the disulfide bond to make the bond more sterically inaccessible. The focus of this work is to examine the effect on relaxivity, degradation, in vivo contrast enhancement, pharmacokinetics, and long-term Gd(III) retention after chemical modification around the disulfide bond.

Structural Modification

Several groups have shown that the reactivity of disulfide bonds change depending on the chemical environment immediately surrounding the disulfide bond (27-29). Table 1 gives the reactivity of several representative disulfide compounds with a free thiol. For each compound that has chemical modification around the disulfide bond, the reactivity is decreased, in some cases by more than an order of magnitude. For example, converting cystamine to cystine decreases the reactivity with HSA Cys-34 from $4.5 \times 10^3$ to $9.3 \times 10^1 \text{ M}^{-1}\text{min}^{-1}$ (28).
Table 1

Reactivity of Several Disulfide Compounds with a Thiol

<table>
<thead>
<tr>
<th>Disulfide Containing Compound</th>
<th>$k$ (M$^{-1}$min$^{-1}$)</th>
<th>Reducing Agent</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="First Disulfide Compound" /></td>
<td>$1.98 \times 10^4$</td>
<td>Cys</td>
<td>29</td>
</tr>
<tr>
<td><img src="image2" alt="Second Disulfide Compound" /></td>
<td>$1.27 \times 10^3$</td>
<td>Cys</td>
<td>29</td>
</tr>
<tr>
<td><img src="image3" alt="Third Disulfide Compound" /></td>
<td>$1.18 \times 10^3$</td>
<td>Cys</td>
<td>29</td>
</tr>
<tr>
<td>$\text{H}_2\text{N-CH}_2-\text{CH}_2-\text{S-S-CH}_2-\text{CH}_2-\text{NH}_2$</td>
<td>$4.5 \times 10^3$</td>
<td>HSA (Cys-34)</td>
<td>28</td>
</tr>
<tr>
<td>$\text{H}_2\text{N-CH-CH}_2-\text{S-S-CH}_2-\text{CH-CH}_2-\text{NH}_2$</td>
<td>$9.3 \times 10^1$</td>
<td>HSA (Cys-34)</td>
<td>28</td>
</tr>
<tr>
<td>$\text{H}_2\text{N-CH-CH}_2-\text{S-S-CH}_2-\text{CH-CH}_2-\text{NH}_2$</td>
<td>$7.6 \times 10^2$</td>
<td>HSA (Cys-34)</td>
<td>28</td>
</tr>
</tbody>
</table>
It would be expected that introducing structural modification around the disulfide bond in the polymeric backbone would modify the degradation rate of the polydisulfides. In this project, modification around the disulfide bond by poly(ethylene glycol) (PEG), shown in Figure 11, was investigated. PEG was selected to modify the chemistry around the disulfide bond because it is nontoxic, nonantigenic, biocompatible, and has a large hydrodynamic volume (30). When conjugated to proteins, PEG was shown to increase distribution of the molecule to the plasma and limit renal filtration (31). PEG grafting to proteins sterically prevents enzymatic biodegradation leading to the observed extended plasma half-life with lower hepatic uptake (30).

For the purposes of this work, we used PEG to increase distribution to the plasma for the length of the exam, but by using smaller PEG lengths (< 2000 Da), when the molecule does degrade it will be readily cleared with minimal tissue retention (32). Therefore, it may hypothesized that PEGylated and biodegradable - via the disulfide bond - macromolecular Gd(III)-based MR contrast agents will be safe as measured by showing acceptable clearance with minimal accumulations and effective by increasing distribution of Gd(III) to the vasculature long enough for high-resolution images for angiographic blood pool contrast-enhanced MR imaging.

\[
\text{HO} - \left(\text{C}_n\text{O}\right) H
\]

Poly(ethylene glycol)

Figure 11. Chemical structure of poly(ethylene glycol).
**Project Aims**

Macromolecular Gd(III) complexes with controllable degradation would have great potential for clinical applications in blood pool contrast enhanced MRI. To evaluate the hypothesis that PEGylation of polydisulfide Gd(III) complexes would increase distribution of Gd(III) to the vasculature long enough for high-resolution images, yet still show acceptable clearance with minimal Gd(III) accumulation, several specific aims were investigated: (1) Synthesize and physiochemically characterize PEGylated biodegradable Gd(III) polymeric contrast agents for MR imaging; (2) Determine relaxation properties and (3) in vitro degradation of the contrast agents; (4) Perform preliminary contrast enhanced MRI with the contrast agents in mice; (5) With a lead agent from the preliminary studies, investigate the pharmacokinetics and biodistribution of the agents; and (6) Investigate the potential for these contrast agents for application in MR angiography (MRA).

**Literature Cited**


Three series of PEG-g-poly(GdDTPA-co-L-cystine) contrast agents were synthesized and characterized in this project. In the first series, monomethoxy poly(ethylene glycol)amine (MPEG-NH₂, MW = 2000 Da) was grafted to GdDTPA-co-L-cystine (GDCP) in a high and low grafting ratio yielding PEG₂₀₀₀ᴴ⁻GDCP and PEG₂₀₀₀ᴸ⁻GDCP, respectively (1). The higher grafting ratio of PEG₂₀₀₀ᴴ⁻GDCP resulted in more prominent enhancement in the blood pool compared to PEG₂₀₀₀ᴸ⁻GDCP (Chapter 5). Consequently, PEG-GDCP of higher grafting degree was selected for future development in the second series of contrast agents. In the second series of PEG-GDCP contrast agents, a high PEG grafting degree was used, but the length of the PEG chain was modified (mPEG-NH₂, MW = 550, 1000, or 2000 Da) yielding PEG⁵₅₀⁻GDCP, PEG¹₀₀₀⁻GDCP, and PEG²₀₀₀⁻GDCP (2). The size of the PEG chain was varied to further characterize the effect that PEGylation has on in vivo contrast enhancement in order to choose an optimal PEG-GDCP contrast agent for larger scale animal studies. PEG₂₀₀₀⁻GDCP resulted in the most extended signal intensity in the blood pool, but it was not chosen for further development since the dynamic contrast enhancement profile of PEG₂₀₀₀⁻GDCP indicated that the clearance may be too restricted via renal filtration, potentially leading to long-term Gd(III) accumulation. PEG¹₀₀₀⁻GDCP also showed
excellent contrast enhancement in the blood pool of mice, yet its dynamic enhancement profile in the kidney was more favorable than that of PEG\textsubscript{2000}-GDCP. Therefore, PEG-1000 was used as the graft for the third series of contrast agents and larger scale animal experiments; it was also tested in three degrees, PEG\textsubscript{1000H}-GDCP, PEG\textsubscript{1000M}-GDCP, and PEG\textsubscript{1000L}-GDCP. This chapter discusses the synthetic approach used for the development of the three different series of PEG grafted biodegradable Gd(III) complexes.

**Methods**

Diethylenetriaminepentaacetic acid (DTPA) was purchased from J. T. Baker (Phillipsburg, NJ). L-Cystine was purchased from Sigma (St. Louis, MO) for the first two series, and from Alfa Aesar (Ward Hill, MA) for the third series of contrast agents. Gd(OAc)\textsubscript{3} was purchased from Alfa Aesar (Ward Hill, MA). MPEG-NH\textsubscript{2} (MW = 2000 Da) for the first series of PEG-GDCP was synthesized according to the literature (3). MPEG-NH\textsubscript{2} (MW = 550, 1000, or 2000 Da) for the second and third series was purchased from Nektar Therapeutics (Huntsville, AL). DTPA-dianhydride (DTPA-DA) was synthesized according to the literature (4). 4-Morpholinepropanesulfonic acid (MOPS) was purchased from Calbiochem® (La Jolla, CA).

**Synthesis of Poly(GdDTPA-co-L-cystine)**

The overall synthetic scheme for the polydisulfide backbone is shown in Figure 12. In general, poly(DTPA-co-L-cystine) (DCP) was synthesized by the copolymerization of L-cystine and diethylenetriaminepentaacetic acid (DTPA) dianhydride followed by complexation with Gd(OAc)\textsubscript{3} yielding poly(GdDTPA-co-L-cystine) (GDCP). Improved synthetic methods, for higher apparent molecular weight and increased Gd(III) complexation efficiency, resulted in three different techniques to synthesize GDCP.
Figure 12. Synthetic scheme of GDCP. (i) Na₂CO₃, H₂O, rt, overnight; (ii) NaOH (aq), -10° to -15° C, 1 hr; (iii) Gd(OAc)₃, H₂O, pH 5-5.5, rt.

Method One

GDCP synthesized by this method served as the backbone for PEG₂₀₀₀.addListener{ GDCP and PEG₂₀₀₀.L-GDCP, the first series of PEGylated contrast agents. L-Cystine (2.40 g, 10 mmol), deionized water (10 ml) and Na₂CO₃ (4.2 g) were stirred for 15 min. DTPA-DA (3.93 g, 11 mmol) was then added in portions to the mixture with stirring. The mixture was stirred overnight at room temperature. Insoluble particles in the reaction mixture were removed by vacuum filtration. The copolymers were dialyzed against deionized water for 24 h using a regenerated cellulose membrane (MWCO = 6000-8000 Da) and were then concentrated in vacuo to dryness. The molecular weights of the copolymers were determined by size exclusion chromatography (SEC) on an AKTA FPLC system.
with a Superose™ 12 (10/300 GL) column with UV and refractive index detectors. The molecular weights were calibrated with poly[N-(2-hydroxypropyl)methacrylamide] standards. The number average \((M_n)\) and weight average \((M_w)\) molecular weights of copolymers were 10.2 and 11.4 kDa, respectively. Proton NMR (ppm, D\(_2\)O): 2.94 (m, 8H, NCH\(_2\)CH\(_2\)N), 3.18 (s, 4H, NCH\(_2\)CONH), 3.25 (d, 2H, NHCHCH\(_2\)S), 3.32 (s, 4H, NCH\(_2\)COOH), 3.48 (s, 2H, NCH\(_2\)COOH), 4.42 (m, 1H, NHCHCH\(_2\)S), please see Figure 13.

Complexation was achieved by reacting poly(DTPA-co-L-cystine) with Gd(OAc)\(_3\) in 8 ml of deionized water. Xylenol orange indicator was added and 1 N HCl was added dropwise until pH 5 - 5.5 was reached. Gd(III) acetate was added to the mixture until the color became pink, indicating an excess of free Gd\(^{3+}\) ions. Excess Gd(OAc)\(_3\) was removed by eluting the solution through a Sephadex G-25 desalting column (Pharmacia). The purified poly(GdDTPA-co-L-cystine) (GDCP) was concentrated to dryness in vacuo and the molecular weights of the copolymers were determined by SEC, \(M_n = 10.0\) kDa, \(M_w = 10.1\) kDa. Gd content was 0.862 mmol Gd/g polymer as determined by ICP-OES (Perkin Elmer, Norwalk, CT, Optima 3100XL).

**Method Two**

GDCP synthesized by the second method (5) was a slight modification of method one. This was the backbone for the second series of contrast agents (varied PEG chain length). DCP was prepared similarly as above. In this case, however, aqueous NaOH solution was used as the base for the copolymerization of L-cystine and DTPA-DA, which was reacted at -10 to -15 °C. First, L-cystine was completely dissolved by adding aqueous NaOH solution dropwise until the pH was ~11-12. Small portions of DTPA-DA
Figure 13. Poly(DTPA-co-L-cystine) $^1$H-NMR spectrum.
were incrementally added and NaOH solution was used to maintain the reaction mixture at pH 11 after the addition of each portion of DTPA-DA. After the reaction, approximately 1 h, the temperature was allowed to increase to room temperature and the pH was decreased to 7-8 by dilute HCl solution. The copolymer was exhaustively dialyzed for 24 h in deionized H₂O. Gd(III) complexation was performed as above and was then fractionated by SEC on an AKTA FPLC with a HiPrep 26/60 column loaded with Sephacryl S-300 beads (Pharmacia, Piscataway, NJ) and eluted with 150 mM NaCl at a rate of 80 mL/h. A fraction of narrow molecular weight distribution GDCP was dialyzed and concentrated to dryness in vacuo (\(M_n = 23.2 \text{kDa}; M_w = 28.1 \text{kDa}; M_w/M_n = 1.2\)) was used for PEG grafting with PEG of different size. Gd(III) contents were determined by ICP-OES to be 0.847 mmol-Gd/g GDCP.

**Method Three**

GDCP synthesized by the third method was used in the third series of experiments with PEG-GDCP contrast agents (PEG-1000, but three different grafting degrees). DCP was synthesized from the copolymerization of DTPA dianhydride and L-cystine described in the second method. Gd(III) complexation was performed by a modified method from the literature (6). Gd(OAc)₃ was added in a 20% molar excess to DCP. The pH of the solution was raised to 10 to precipitate out free Gd and centrifuged at 4000 rpm for 20 min. The supernatent was dialyzed in sodium citrate buffer (pH 6.5, 0.1 M, 4-5 buffer changes) overnight with a regenerated cellulose membrane (MWCO = 6000-8000 Da). The solution was then dialyzed by exhaustively changing ultrapure water for 48. The polymer solution was then concentrated to an oil and freeze-dried for 96 h, \(M_n = \)
37.8 kDa; \( M_w = 54.2 \) kDa; \( M_w/M_n = 1.4 \); 1.20 mmol-Gd/g polymer. This polymer was used for PEGylation of the third series of contrast agents.

For the third series of PEG-GDCP contrast agents, a second batch of GDCP of the desired molecular weight was prepared to match the molecular weight of PEG-GDCP. This was achieved by fractionation using size exclusion chromatography with a Superose™ 6 preparative grade XK 50/100 column; 0.5 g GDCP was loaded per fractionation. The elution rate was 7.5 ml/min with 0.02 TRIS buffer (pH 7.4). The polymer fractions were desalted by exhaustive dialysis, concentrated to a thick, clear, sticky oil and freeze-dried for 96 h. The weight average \( (M_w) \) and number average \( (M_n) \) molecular weight of the fractions were determined using an AKTA FPLC system equipped with a Superose™ 6 10/300 GL analytical column and UV and refractive index detectors. The system was calibrated by poly[N-(2-hydroxypropyl)methacrylamide] standards. Fractionated GDCP resulted in a contrast agent with narrow molecular weight distribution \( (M_w = 43.3, M_w/M_n = 1.1) \); Gd content 1.084 mmol-Gd/g polymer.

**Synthesis of PEG2000L-g-poly(GdDTPA-co-L-cystine)**

GDCP (200 mg, 0.26 mmol cystine) was dissolved in 4 ml deionized water and N-hydroxysuccinimide (270 mg, 2.4 mmol) was then added with stirring. An excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (700 mg, 3.65 mmol) was slowly added and the resulting yellow solution was stirred for 15 min. MPEG-NH₂ (MW = 2000 Da, 262 mg) was added in portions and the reaction mixture was stirred overnight (Figure 14). White precipitate was removed by filtration. Unreacted PEG and salts were removed by ultrafiltration using a Centricon-10 concentrator (membrane MWCO = 10,000 Da). The resulting PEGylated poly(GdDTPA-co-L-cystine) (PEG2000L-GDCP)
Figure 14. Synthetic scheme of PEG-GDCP.

was characterized by SEC and ICP-OES as described above. PEG content on the graft copolymers was determined from the difference between the weight of the sample and the amount of GDCP in the sample based on Gd content given by ICP-OES and the molecular weight of the PEG chain in the graft copolymers. PEG2000L-GDCP: $M_n = 21.0$ kDa, $M_w = 24.5$ kDa; 0.720 mmol-Gd/g polymer; PEG/Gd = 0.33.

Synthesis of PEG2000H-g-poly(GdDTPA-co-L-cystine)

PEG-g-poly(GdDTPA-co-L-cystine) (PEG2000H-GDCP) with high PEG content was prepared similarly with a high PEG to GDCP ratio. Briefly, poly(GdDTPA-co-L-cystine) (200 mg, 0.26 mmol cystine) was dissolved in 4 ml deionized water, and $N$-hydroxysuccinimide (270 mg, 2.4 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (700 mg, 3.65 mmol) was then added with stirring. MPEG-NH$_2$ (MW = 20000, 524 mg) was added to the reaction and the mixture was stirred overnight. Unreacted PEG and salts were removed by ultrafiltration using a Centricon-10 concentrator (membrane MWCO = 10,000 Da). PEG2000H-GDCP: $M_n = 19.9$ kDa, $M_w = 22.9$ kDa; 0.449 mmol-Gd/g polymer; PEG/Gd = 0.76.
Synthesis of PEG\textsubscript{n}-g-poly(GdDTPA-co-L-cystine)

PEG\textsubscript{2000}-g-poly(GdDTPA-co-L-cystine) (PEG\textsubscript{2000}-GDCP) was synthesized from the 1:1 stoichiometric ratio of GDCP (100 mg, 0.13 mmol cystine) from the second method to MPEG-NH\textsubscript{2} (MW = 2000 g/mol, 266 mg, 0.13 mmol) via NHS (152.8 mg, 1.3 mmol) and EDC (381.9 mg, 2.0 mmol). GDCP was first dissolved in 2 ml of DI H\textsubscript{2}O with stirring. NHS and EDC were then added to the solution and allowed to stir for 20 min. MPEG-NH\textsubscript{2}-2000 was added slowly to the mixture. The reaction was stirred overnight at room temperature. Excess PEG was then removed by ultrafiltration using a Centricon-10 concentrator (membrane MWCO = 10,000 Da). Purified PEG\textsubscript{2000}-GDCP graft copolymers were concentrated to dryness. PEG\textsubscript{1000}-g-poly(GdDTPA-co-L-cystine) (PEG\textsubscript{1000}-GDCP) and PEG\textsubscript{550}-g-poly(GdDTPA-co-L-cystine) (PEG\textsubscript{550}-GDCP) were prepared and purified by using the same procedure with the same PEG/cystine molar ratio. The purified PEGylated GDCP copolymers were characterized by SEC and gadolinium contents were determined by ICP-OES (Perkin-Elmer, Norwalk, CT, Optima 3100 XL). PEG\textsubscript{2000}-GDCP: \( M_n = 28.5 \) kDa, \( M_w = 37.7 \) kDa, Gd content = 0.321 mmol Gd/g polymer, PEG/Gd = 1.18. PEG\textsubscript{1000}-GDCP: \( M_n = 31.1 \) kDa, \( M_w = 37.8 \) kDa, Gd content = 0.565 mmol Gd/g polymer, PEG/Gd = 1.02. PEG\textsubscript{550}-GDCP: \( M_n = 30.8 \) kDa, \( M_w = 33.7 \) kDa, Gd content = 0.615 mmol Gd/g polymer, PEG/Gd = 1.30.

Synthesis of PEG\textsubscript{1000x}-g-poly(GdDTPA-co-L-cystine)

PEG\textsubscript{1000x}-GDCP of varying PEG grating degree was synthesized similarly as reported for PEG\textsubscript{2000x}-GDCP (23). Briefly, MPEG-NH\textsubscript{2} (MW = 1000 Da) was conjugated to GDCP from the third method that was not fractionated (MW = 54.2 kDa, Mw/Mn = 1.4) via NHS and EDC chemistry in one of three stoichiometric ratios; PEG:L-
cystine = 1.0, PEG:L-cystine = 0.5, PEG:L-cystine = 0.2. PEG that did not react was
removed from the reaction using a Millipore ultrafiltration system fitted with a
regenerated cellulose membrane (MWCO = 10,000 Da). PEGylated contrast agents were
characterized by SEC and ICP-OES (Optima 3100XL, PerkinElmer, Boston, MA).
PEG1000H-GDCP: $M_n = 35.8$ kDa, $M_w = 42.1$ kDa, Gd content = 0.568 mmol-Gd/g
polymer, PEG/Gd = 0.96. PEG1000M-GDCP: $M_n = 38.9$ kDa, $M_w = 46.5$ kDa, Gd content
= 0.700 mmol-Gd/g polymer, PEG/Gd = 0.65. PEG1000L-GDCP: $M_n = 45.7$ kDa, $M_w =
52.1$ kDa, Gd content = 0.861 mmol-Gd/g polymer, PEG/Gd = 0.39.

Relaxivity

For the first two series of contrast agents, the $T_1$ relaxivity for the novel polymeric
contrast agents was determined on a Siemens Trio 3T MR scanner. $T_1$ relaxation times
for four concentrations of each contrast agent were determined by the sequential
application of a standard inversion-recovery (IR) pulse sequence with $TR = 5000$ ms, $TE
= 17$ ms for inversion times $TI = 22, 50, 70, 100, 200, 300, 400, 500, 600, 700, 800, 900,
1000, 2500, and 3500 ms in a water bath at room temperature, 21°C. Net magnetization
amplitude data for each sample was determined from the appropriate region of interest
(ROI), using OsiriX software (http://homepage.mac.com/rossetantoine/osirix/Index2.
html), and fit to the following multiparametric nonlinear regression (eq 12):

$$M_{TI} = M_0 \left[ 1 - 2 \exp \left( -\frac{1}{T_1 \cdot TI} \right) \right]$$  \hspace{1cm} [12]

where $TI$ is the inversion time, $M_{TI}$ is the net amplitude of a selected ROI at time, $TI$. The
maximum amplitude at $TI = 0$, $M_0$, and $T_1$, the concentration dependent relaxation time of
the agent are fitted parameters. $M_0$ and $T_1$ were determined simultaneously for each
concentration of contrast agent using Matlab® (The Mathworks, Inc., Natick, MA) software. Plotting $1/T_1$ vs. [Gd(III)] and taking the slope of the linear equation for each polymer then gave the relaxivity, $r_1$.

For the third series of contrast agents, the longitudinal relaxation rates for the three PEG modified GDCP contrast agents and GDCP were determined on Siemens TrioTim 3T MR scanner. A birdcage headcoil was used for signal detection. Four concentrations of each contrast agent in MOPS buffer (pH 7.4) were imaged with an inversion recovery (IR) pulse sequence to determine $T_1$ relaxation rates. Imaging parameters for the IR pulse sequence were $TE = 17$ ms, $TR = 5000$ ms, 150° flip angle, and inversion times ($TI$) of 25, 35, 50, 75, 100, 200, 400, 800, and 1600 ms. $T_1$ relativities were calculated using Matlab® (The Mathworks, Inc., MA) software. Regions-of-interest were placed in the center of each vial and the signal intensity versus $TI$ for each vial was fit to a parametric equation yielding $M_0$ and $1/T_1$. The slope of the linear equation of $1/T_1$ and Gd(III) concentration gave the resulting $T_1$ relaxivity.

**Results and Discussion**

A schematic of the synthetic procedure of GDCP and PEG-GDCP is described in Figures 12 and 14. Physiochemical parameters are shown in Table 2. Poly(DTPA-co-L-cystine) (DCP) was first synthesized by the copolymerization of DTPA dianhydride with L-cystine. L-Cystine is sparingly soluble in organic solvents so the copolymerization was performed in basic aqueous conditions. In the first method of GDCP synthesis, the weak base Na$_2$CO$_3$ was used to maintain a relatively basic pH and to minimize the hydrolysis of the dianhydride. The molecular weight of DCP was relatively low due to partial hydrolysis of the dianhydride, limiting chain growth of the copolymers.
In the second and third series, a modified method using aqueous NaOH solution increased the molecular weight of GDCP. Using this method completely solublized L-cystine to react with DTPA-DA. The reaction was kept at a lower temperature (-15 to -10 °C), which may have lowered the rate of anhydride hydrolysis. Using a saturated NaOH solution kept the reaction volume at a minimum and thus reactant concentration at a maximum. In addition, the reaction time was 1 h, compared to overnight for the thirst method. In the first two syntheses, GDCP was dried on a gas vacuum pump, whereas the third was freeze-dried. The more effective freeze-drying method most likely accounted for the higher Gd content in the third series of GDCP because water more efficiently removed. Because improvements were made with each modified synthetic method of GDCP, the backbone molecular weight for each PEG-GDCP was increased. The overall effect of increasing the backbone length does not contribute significantly to blood pool contrast enhancement. This will be further discussed in Chapter 5.

In the first series of PEGylation, MPEG-NH$_2$ with an average molecular weight of approximately 2000 Da was used in the modification of GDCP (method one). The content of PEG in PEG-g-poly(GdDTPA-co-L-cystine) was controlled by the molar ratio of MPEG-NH$_2$ and GDCP. PEG-g-poly(GdDTPA-co-L-cystine) with two different degrees of PEG modification were prepared. The molar ratio of PEG to the Gd-DTPA monomer was 0.33 and 0.76 for conjugates PEG$_{2000L}$-GDCP and PEG$_{2000H}$-GDCP, respectively, calculated from the Gd content per gram of polymer sample before and after PEGylation as determined by ICP-OES. The ratio of PEG grafted to cystine residues in the GDCP backbone is lower than the stoichiometric ratios used in the reaction, possibly due to the spatial restriction caused by the large hydrodynamic radius of PEG blocking
the conjugation of more PEG molecules to the adjacent cystine residues. PEGylation increased the hydrodynamic volume of the copolymers. However, increasing PEG content did not result in further increase of the hydrodynamic volume of the copolymers when comparing PEG\textsubscript{2000L}-GDCP ($M_w = 24.5$ kDa) with PEG\textsubscript{2000H}-GDCP ($M_w = 22.9$ kDa).

For the second series of PEG-GDCP contrast agents, PEG\textsubscript{2000}-GDCP, PEG\textsubscript{1000}-GDCP and PEG\textsubscript{550}-GDCP, GDCP (method two) was first fractionated and a fraction with narrow molecular weight distribution ($M_n = 23.2$ KDa; $M_w = 28.1$ KDa) was used for modification with PEG. The improved synthetic method GDCP increased the backbone length of GDCP, which is advantageous because renal clearance decreases above 20 kDa (7). Monomethoxy-PEG amine with three different molecular weights, 2000, 1000, and 550 Da were grafted onto the GDCP backbone. Each PEG graft was synthesized in a ratio of one PEG molecule to one L-cystine molecule. For each PEG grafting, the molecular weight distribution of the copolymers shifted to a higher molecular weight after the reaction and unreacted PEG was removed by ultrafiltration, as shown by SEC.

The apparent molecular weight of PEG\textsubscript{2000}-GDCP ($M_w = 37.7$ kDa) and PEG\textsubscript{1000}-GDCP ($M_w = 37.8$ kDa) were similar and it was slightly smaller for PEG\textsubscript{550}-GDCP ($M_w = 33.7$ kDa). These apparent molecular weight of these agents was also calculated based on a protein standard curve. Using protein standards, the apparent molecular weights of the contrast agents were $M_w = 92.2$, 92.1 and 84.7 kDa for PEG\textsubscript{2000}-GDCP, PEG\textsubscript{1000}-GDCP, and PEG\textsubscript{550}-GDCP, respectively. All three polymers had PEG/Gd molar ratios slightly greater than one (PEG\textsubscript{2000}-GDCP, 1.18; PEG\textsubscript{1000}-GDCP, 1.02; PEG\textsubscript{550}-GDCP, 1.30). Based on the number average molecular weight of GDCP, there are approximately 30
Gd(III) chelate units per polymeric contrast agent and 35 to 40 PEG chains grafted to GDCP. The physicochemical parameters of the copolymers are summarized in Table 2.

In the third generation of PEG-GDCP, GDCP fractionated with size exclusion chromatography (method three) resulted in a contrast agent with narrow molecular weight distribution ($M_w = 43.3$, $M_w/M_n = 1.1$), higher than in the second series. This was done to match the molecular weight range of PEG-GDCP that was synthesized from GDCP that had not been previously fractionated. The conjugation of PEG to the GDCP polymeric precursor resulted in macromolecular Gd(III) complexes that were of similar size and polydispersity compared to fractionated GDCP (Table 2). The molecular weight of PEG-GDCP with the highest PEG ratio, PEG$_{1000H}$-GDCP, resulted in a PEG grafted polymer with the lowest molecular weight ($M_w = 42.1$ kDa) of the three PEG-GDCP contrast agents. PEG$_{1000M}$-GDCP (medium PEG grafting degree) had a slightly higher molecular weight ($M_w = 46.5$), while PEG$_{1000L}$-GDCP (low PEG grafting degree) had the highest molecular weight ($M_w = 52.1$ kDa) of the three PEG-GDCP contrast agents. This is consistent with previous PEG-GDCP results in the first series of PEG-GDCP where the grafting degree was varied and is likely due to more conversion of negatively charged GDCP repeat units into neutral amide linkages with increasing PEG grafting degree.

Grafting PEG-1000 to GDCP in the highest degree resulted in PEGylation of nearly every repeat unit (PEG/Gd = 0.96). This is slightly lower than the theoretical ratio PEG/Gd ratio of 1.0. Most likely, grafting a PEG chain to every backbone unit is sterically restrictive. Grafting of PEG in the medium and low degree resulted in PEGylation of 65% and 39% of the repeat GDCP units, slightly higher than the stoichiometric ratios of the reaction.
<table>
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<tr>
<th>Contrast Agent</th>
<th>$M_w$ (kDa)</th>
<th>$M_n$ (kDa)</th>
<th>$M_w/M_n$</th>
<th>Gd-content (mmol/g polymer)</th>
<th>PEG/Gd</th>
<th>$T_1$-Relaxivity (mM$^{-1}$s$^{-1}$)</th>
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<tr>
<td>GDCP$^a$</td>
<td>10.1</td>
<td>10.0</td>
<td>1.01</td>
<td>0.862</td>
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<td>GDCP$^b$</td>
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<td>23.1</td>
<td>1.22</td>
<td>0.847</td>
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<tr>
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<td>38.7</td>
<td>1.40</td>
<td>1.084</td>
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<td>6.1</td>
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<tr>
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<td>40.0</td>
<td>1.08</td>
<td>1.084</td>
<td>-</td>
<td>6.1</td>
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<td>PEG$_{2000H}$-GDCP</td>
<td>22.9</td>
<td>19.9</td>
<td>1.15</td>
<td>0.449</td>
<td>0.76</td>
<td>12.7</td>
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<td>0.321</td>
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<td>0.700</td>
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<td>1.15</td>
<td>0.861</td>
<td>0.39</td>
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$^a$Used to synthesize PEG$_{2000H}$-GDCP and PEG$_{2000L}$-GDCP.

$^b$Used to synthesize PEG$_{2000}$-GDCP, PEG$_{1000}$-GDCP, and PEG$_{550}$-GDCP.

$^c$Used to synthesize PEG$_{1000H}$-GDCP, PEG$_{1000M}$-GDCP, PEG$_{1000L}$-GDCP.
The results of the relaxivity study are shown in Table 2. In general, there does not appear to be any specific trend for PEGylation effects on relaxivity. Both PEGylated polymers, PEG$_{2000L}$-GDCP and PEG$_{2000H}$-GDCP, had a higher relaxivity than GDCP (11.2 mM$^{-1}$s$^{-1}$), while PEG$_{2000L}$-GDCP had a higher relaxivity than PEG$_{2000H}$-GDCP, 16.3 and 12.7 mM$^{-1}$s$^{-1}$, respectively. It is likely that PEG-2000 offers some steric limitation to the movement of the polymer, which increases the rotational correlation coefficient of the copolymers and in turn the relaxivity (8). However, PEG$_{2000H}$-GDCP with high PEG content possessed lower relaxivity than PEG$_{2000L}$-GDCP with low PEG content. These results are consistent with the literature. Raymond et al. suggested that this is due to the possible association of water to PEG (9). Increased PEG content increased the amount of water associated to PEG via hydrogen bonding, which may interfere with the interaction of Gd(III) ions and water. Potentially, this causes a decrease in the water-ion exchange rate, which increases the residence time of the coordinated water, $\tau_M$, resulting in a decreased relaxivity, as shown in eq 13:

$$R_{1p}^{is} = \frac{cq}{55.6} \cdot \frac{1}{T_{1M}^H + \tau_M}$$

[13]

where $R_{1p}^{is}$ is the inner coordination sphere relaxation of bulk water surrounding the paramagnetic ion; $c$ is the concentration of the ion; $q$ is the number of coordinated water molecules to the metal ion; and $T_{1M}^H$ is the longitudinal relaxation time of the coordinated water (10).

The relaxivity of the PEG$_{n}$-poly(GdDTPA-co-L-cystine) did not change significantly as compared to their precursor, GDCP, which has a $T_1$ relaxivity of 8.31 mM$^{-1}$sec$^{-1}$ (based on Gd concentration) at 3 Tesla. When PEG-2000 was grafted the
relaxivity increased marginally to 8.73 mM⁻¹s⁻¹. For PEG₁₀₀₀-GDCP and PEG₅₅₀-GDCP the relaxivity decreased slightly to 7.79 and 7.83 mM⁻¹s⁻¹, respectively. The $T_1$ relaxivity of PEG₂₀₀₀-GDCP was higher than that of PEG₁₀₀₀-GDCP and PEG₅₅₀-GDCP.

The impact of PEG modification with different sizes on the $T_1$ relaxivity of the contrast agents was moderate. The modified agents had similar $T_1$ relaxivity as their precursor, GDCP (8.31 mM⁻¹s⁻¹). PEG₂₀₀₀-GDCP (8.73 mM⁻¹s⁻¹) has slightly higher relaxivity than PEG₁₀₀₀-GDCP (7.79 mM⁻¹s⁻¹) and PEG₅₅₀-GDCP (7.83 mM⁻¹s⁻¹). The relaxivity of these agents is lower than that of the agents with lower grafting degrees that were synthesized in the first series of contrast agents. The $T_1$ relaxivity of PEG₂₀₀₀-GDCP with grafting ratios of 0.33, 0.76 and 1.2 is 16.3, 12.7 and 8.73 mM⁻¹s⁻¹, respectively. It appears that the increase of PEG grafting degree in the copolymer chains decreases the $T_1$ relaxivity of the agent. A plausible explanation is that a high degree of PEG on the polymer chains may interfere with the interaction of water molecules with the Gd(III) complexes because PEG can associate with water molecules via hydrogen bonding (8).

For the third series of PEG-GDCP, the $T_1$ and $T_2$ relaxivity of each the contrast agents was measured at 3 T in MOPS buffer at physiological pH. The longitudinal relaxivity of GDCP, 6.1 mM⁻¹s⁻¹, was slightly higher than any of the PEG-GDCP contrast agents. As was the case PEG₂₀₀₀-GDCP at higher grafting degrees, the relaxivity was relatively lower for PEG₁₀₀₀-GDCP of the third series of contrast agents. However, further decreasing the grafting degree with PEG₁₀₀₀-GDCP did not increase the relaxivity. One possible explanation is that PEG-2000 may offer some structural resistance to
motion that PEG-1000 does not, which would explain why PEG-2000 grafting affected relaxivity and PEG-1000 does not seem to have this effect.

Summary

When PEG was grafted to GDCP in the first two series of contrast agents, it increased the apparent molecular weight of the macromolecular agent. It is interesting to note that the amount of MPEG-NH₂ grafted on the GDCP did not significantly affect the apparent molecular weight of the grafted agent for any of the three series of contrast agents. The increase of hydrodynamic volume of PEG₂₀₀₀H-GDCP with a high PEG to Gd ratio (approximately 0.76:1) was less appreciable than that of PEG₂₀₀₀L-GDCP with a low ratio (approximately 0.33:1). Although this seems counterintuitive because of PEG's large hydrodynamic radius, it is plausible that when more PEG is grafted, the density of PEG molecules on the polymer chain increases and it does not extend the length of the copolymers that have a fixed length. The higher PEG density along the polymer chain may increase intermolecular interaction between the PEG chains and, probably, diminish their contribution to the hydrodynamic volume of the macromolecules.

It was also informative to know how the size of PEG affects the physicochemical properties and in vivo contrast enhancement (Chapter 5) of the modified agents. In the second series of contrast agent to minimize the potential variations due to the wide molecular distribution of GDCP copolymers, the copolymers were fractionated and the same narrow fraction (M_w = 28.2 KDa, M_w/M_n = 1.2) was used for grafting with PEG of different molecular weights. PEG modified GDCP has approximately one PEG molecule per repeat unit regardless of the size of PEG. The apparent molecular weight of the copolymers increased after the modification. In addition, little difference was observed
for the relaxivity of PEG grafted to GDCP at high ratios, independent of PEG molecular weight.

In the third series of polymers, PEG grafting to GDCP resulted in grafted copolymers of narrow molecular weight distribution but slightly different apparent molecular weights. PEG$_{1000}$-GDCP, which had the lowest PEG grafting degree (PEG/Gd = 0.39), had a molecular weight of 52.1 kDa that slightly lower than the GDCP that it was synthesized from (54.2 kDa). Increasing the PEG content to a medium degree (PEG/Gd = 0.65) resulted in a contrast agent with a molecular weight of 46.5 kDa. Further increasing the degree of PEG grafting (PEG/Gd = 0.96) resulted in a further decrease of the apparent molecular weight, 42.1 kDa. These results are consistent to those obtained for GDCP modified with PEG-2000 of varying grating degree, where apparent molecular weight decreased with increased grafting degree. Increased PEG grafting degree results in more neutral amide bonds compared to more free carboxylate groups of PEG-GDCP with lower PEG grafting degree. Therefore increased PEG content may actually reduce the overall hydrodynamic size of the contrast agents.

**Literature Cited**


CHAPTER 4

IN VITRO DEGRADATION

The expectation that PEGylated polydisulfide-based Gd(III) complexes are biodegradable is a major premise to the success of this project. The goal of this project is to synthesize macromolecular Gd(III) complexes that initially distribute to the vascular pool and then after a reasonable period of time for an MRI examination, break down into small molecules or oligomers that can be readily cleared by renal filtration with minimal tissue retention of Gd(III). If this is to occur, it is of great importance to verify the potential of polydisulfide-based contrast agents to break down via the thiol-disulfide exchange reaction. In this chapter, the methods to examine in vitro degradability, results with two series of PEG-GDCP contrast agents, and a discussion, including other biodegradable macromolecular polydisulfide contrast agents will be presented.

Methods

PEGylated polymers PEG_{2000L}-GDCP and PEG_{2000H}-GDCP (Table 2, page 71) were separately incubated in 100 µM aqueous cysteine solution at 37°C (I). The higher thiol concentration compared to plasma conditions was used to demonstrate the feasibility of degradation via the thiol-disulfide exchange reaction. Size exclusion chromatography (SEC) on an AKTA FPLC system with a Superose™ 12 (10/300 GL) column with UV and refractive index detectors was performed on the samples at 0, 3 and 24 h after
incubation to demonstrate the time dependent degradation of the disulfide bonds by monitoring the molecular weight change of the copolymers. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was then performed on a reaction mixture after an excess of L-cysteine was added to observe the degradation products of PEG\textsubscript{2000L}-GDCP and PEG\textsubscript{2000H}-GDCP.

In a second study, PEG\textsubscript{2000}-GDCP, PEG\textsubscript{1000}-GDCP, and PEG\textsubscript{550}-GDCP (0.64 mM cystine based on Gd(III) content of the copolymers) were separately incubated in an aqueous cysteine solution at varying concentrations of cysteine (10, 100, 1,000, 10,000 µM) for 1 h at 37°C (2). Since the in vitro study was performed to explore only relative differences between the three PEG-GDCP contrast agents of the second series, a range of thiol concentrations was selected to accelerate the degradation process to identify differences between these three contrast agents. The incubation mixture was analyzed by SEC with a UV detector to examine the effect of thiol concentration and PEG chain length on the degradation of the polymeric contrast agent. An in vitro degradation study was not performed on third series of PEG-GDCP contrast agents, because extensive degradation studies were performed for on a wide variety of PEG-GDCP contrast agents in the first two series of PEG-GDCP contrast agents. In addition, the emphasis of the third series of contrast agents was in vivo pharmacokinetics and long-term Gd(III) tissue accumulation.

Results

The in vitro degradation study was performed by incubating the macromolecular agent with L-cysteine to verify the degradability of the agents via the thiol-disulfide exchange reaction. The results showed the expected decrease for the high molecular
weight fraction and an increase in PEGylated degradation products of the agents in the incubation mixture. SEC analysis of the incubation mixtures showed that there was a significant shift of molecular weight distribution to lower molecular weight, representing a breakdown in the most prominent polymer species. The time dependent molecular weight distribution of PEG\textsubscript{2000L}-GDCP and PEG\textsubscript{2000H}-GDCP in the incubation is shown in Figure 15.

![Graph a](image1.png)

**Figure 15.** In vitro degradation of PEG\textsubscript{2000X}-GDCP. The effect of L-cysteine (100 µM) on the degradation of PEG\textsubscript{2000L}-GDCP (a) and PEG\textsubscript{2000H}-GDCP (b) at 37 °C.
In Figure 15, the initial polymer peak for PEG\(_{2000L}\)GDCP at 27.4 min completely disappeared after 24 h of incubation. Consequently, there is a rise in signal intensity at 31.4 and 34.8 min over the same time period representing an increase in the PEGylated and unPEGylated degradation units, respectively. On the other hand the degradation PEG\(_{2000H}\)GDCP was considerably less prominent in the presence of L-cysteine at 3 h compared to PEG\(_{2000L}\)GDCP (Figure 15b). After 24 h, there was no further degradation of PEG\(_{2000H}\)GDCP. The difference between the 24 h peaks of PEG\(_{2000H}\)GDCP and PEG\(_{2000L}\)GDCP indicate that PEG\(_{2000L}\)GDCP is more susceptible to degradation to the thiol-disulfide exchange reaction in vitro than GDCP with a higher PEG-2000 grafting degree. The low molecular weight degradation complexes I, II, and III in Figure 16 were identified in the MALDI-TOF mass spectrum acquired on the reaction mixture of an excess of L-cysteine and both PEG\(_{2000H}\) and PEG\(_{2000L}\)GDCP, shown in Figure 17.

PEG\(_{2000}\)GDCP, PEG\(_{1000}\)GDCP, and PEG\(_{550}\)GDCP were incubated with various concentrations of L-cysteine to verify the degradability of the copolymers via the thiol-disulfide exchange reaction and to study the effect of PEG chain length on the degradation. Figure 18 shows that for all three polymeric contrast agents, a low concentration of cysteine (10 \(\mu\)M) had little effect on the molecular weight distribution of each polymer after one hour. As the concentration of L-cysteine increased, the fraction of high molecular weight polymers decreased while the fraction of low molecular weight PEGylated degradation units increased. Reduction in the high molecular weight fraction was the least for PEG\(_{2000}\)GDCP. In comparison, PEG\(_{550}\)GDCP had a more rapid decrease from the high molecular weight fractions to a more dramatic increase in the low
Figure 16. PEG-GDCP degradation products. Degradation products identified in the MALDI-TOF MS spectrum after the incubation of PEG_{2000x}-GDCP with L-cysteine.

\[ R = \text{OH or HN-PEGm} \]
Figure 17. MALDI-TOF MS Spectrum PEG2000-GDCP. Resulting MALDI-TOF MS of PEG2000L-GDCP (a) and PEG2000H-GDCP (b) incubation mixtures.
Figure 18. Degradation of PEGₙ-GDCP. The molecular weight distribution of PEG₂₀₀₀-GDCP (a), PEG₁₀₀₀-GDCP (b), and PEG₅₅₀-GDCP (c) with the incubation of varying concentrations of L-cysteine solutions for 1 h.
molecular weight fractions. PEG\textsubscript{1000}-GDCP also had a significant increase in the low molecular weight fraction, but not as substantial as PEG\textsubscript{550}-GDCP. It appears that the length of PEG affects the degradation rate though the thiol-disulfide exchange reaction.

**Discussion**

The first series of PEG-GDCP contrast agents, PEG\textsubscript{2000H}-GDCP and PEG\textsubscript{2000L}-GDCP, showed that degradation was dependent on PEG grafting density in vitro. Based on preliminary imaging results in vivo (Chapter 5), PEG-GDCP with high grafting density was chosen for the second series of PEG-GDCP contrast agents, where the length of the PEG chain was modified. From the degradation study with the first series of PEG-GDCP, it was known that the degradation of high grafting ratio PEG-GDCP was quite slow. Therefore, in the second series, increased concentrations of a free thiol were used to demonstrate susceptibility as the marker of biodegradability. As expected, PEG-GDCP with the longest PEG chain length was least susceptible to degradation, whereas PEG-GDCP with the shortest PEG chain was the most susceptible. The results of these two studies suggest that at least in a controlled in vitro environment, PEG appears to cause some shielding of the disulfide bond to free thiols.

In the literature, adding side groups around the disulfide bond of small molecules results in a decrease of the thiol-disulfide exchange rate Table 1 (3,4). The chemical structure of polydisulfide Gd(III) complexes developed by this group is shown in Figure 19. GDCC showed a rapid degradation via disulfide reduction to a thiol in vitro (5). When the structure is modified to an ester, this degradation rate is decreased (6), but the degradation rate was shown to increase if a small amide is introduced around the disulfide bond (7). Within the group of amide modifications of GDCC, however, the
rapid degradation is least severe with GCIC, which has the most sterically bulky substituent. Interestingly, GDCP itself shows little degradation to a free thiol in vitro. It may be hypothesized that because GDCP is a polyanion, there may be electrostatic repulsion to a thiolate anion, which would be in equilibrium with a thiol at physiological pH (8). These results were supported by a rat plasma experiment where GDCEP degraded more rapidly than GDCP over the course of 30 min (9).

In summary, degradation of polydisulfides can be tuned to some degree, but the mechanisms are complex and further study is required. In vitro PEGylated GDCP, whether modified in PEG grafting degree or PEG chain length, degraded more slowly than GDCC (R = H). It was shown if GDCC is modified with a small amide, the degradation rate actually increases, suggesting that the disulfide bond may be destabilized by a proximal amide. Even though the conjugation of PEG to GDCP results in an amide linkage, the degradation rate decreases compared to GDCC in vitro. It is likely that the much larger PEG may protect the disulfide bond, at least in vitro, even if there is a
destabilizing amide present. Therefore if the amide bond destabilizes the disulfide linkage, this effect may not be detectible in vitro for PEG-GDCP.

Literature Cited


CHAPTER 5

IN VIVO CONTRAST ENHANCED MRI

The first two series of biodegradable PEG-GDCP contrast agents, PEG$_{2000x}$-GDCP and PEG$_n$-GDCP, were tested in vivo for their efficacy at enhancing MR images. The goals of these first two in vivo MRI studies were to examine the effect of PEG grafting degree ($I$) and PEG chain length ($2$) on signal enhancement, including the dynamic blood pool signal and relative distribution of signal intensity in various tissues. From the combined results of the first two studies, a lead agent was selected for pharmacokinetic, biodistribution and preliminary MR angiography studies (Chapters 6 and 7). The methods, results, and discussion, including rationale for selecting contrast agents for each subsequent series, are presented in this chapter.

Methods

All animals involved in the experiments shown here were performed under an approved protocol from the University of Utah Institutional Animal Care and Use Committee.

MRI Protocol

Prior to MR imaging, healthy mice were anesthetized by either an intramuscular or interperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg). MR imaging
was performed on a Siemens Trio 3T MR scanner with a wrist receiver coil. The mice were placed in the center of the wrist coil and images were obtained using a 3D FLASH pulse sequence. The temperature of each mouse was maintained between imaging sequences using a warming pad.

Contrast enhancement studies using biodegradable macromolecular contrast agents with a varying PEG grafting degree, the first series of PEG-GDCP contrast agents (PEG_{2000x}-GDCP), were investigated in CD-1 mice (Charles River Laboratories Wilmington, MA). Polymeric contrast agents, including PEG_{2000L}-GDCP, PEG_{2000H}-GDCP, and GDCP, were administered by an intravascular bolus injection at a dose of 0.03 mmol-Gd/kg into a tail vein of the mice. Please see Table 2 for the physiochemical parameters of these agents. The clinical control contrast agent, Gd(DTPA-BMA), was similarly administered at a dose of 0.1 mmol Gd/kg. Pre- and postcontrast imaging parameters were $TE = 1.74$ ms, $TR = 4.3$ ms, 25° rf tip angle, 3D acquisition with 128 slices, 120 mm FOV, and 1.6 mm coronal slice thickness. Images were acquired before and at 1, 5, 15, 30, and 60 min postinjection of contrast agent. Each agent was investigated in a group of four mice.

A second MRI pulse sequence was used for imaging of PEG-GDCP agents with varying length of PEG chain. The imaging parameters were as follows: $TE = 2.4$ ms, $TR = 7.4$ ms, 25° rf tip angle, 3D acquisition with 64 slices, 120 mm FOV, and 0.5 mm coronal slice thickness. Each 3D data set took approximately 1 min 40 sec to acquire. PEG_{2000}-GDCP, PEG_{1000}-GDCP, and PEG_{550}-GDCP were administered by an intravenous bolus injection at a dose of 0.05 mmol-Gd/kg into the tail vein of female nu/nu athymic
mice. Images were acquired prior to injection and at 2, 5, 10, 15, 30, and 60 min postinjection of the contrast agents. Each agent was tested in a group of three mice.

Image Processing

Raw MR images were transferred to an Apple Macintosh workstation running OS X. The images were analyzed using OsiriX, an open-source DICOM image processing software package (http://homepage.mac.com/rossetantoine/osirix/). Images were analyzed for the depiction of major organs as well as fine blood vessels and the duration of the enhancement from each agent. Contrast enhancement for the second series of PEG-GDCP contrast agents was semi-quantitatively measured by taking the signal intensity in the tissue of interest at time \( t \), \( \text{Signal}_{\text{tissue}}(t) \) relative to the signal in a thigh muscle at time \( t = 0 \), \( \text{Signal}_{\text{muscle}}(0) \) as shown in the following calculation (eq 14):

\[
\text{Signal Intensity Ratio } (t) = \frac{\text{Signal}_{\text{tissue}}(t)}{\text{Signal}_{\text{muscle}}(0)} \tag{14}
\]

Statistical analysis of the MR data was performed using GraphPad Prism software. The MR signal intensity ratio at each time point is the average from three different mice. The standard deviation of the signal intensity ratio was also determined for each time point. Statistical differences between polymeric contrast agents at each data point for a specific tissue were calculated using a one-way ANOVA with a Tukey posttest to determine which pair of data points accounted for the statistical difference in the grouping of the three contrast agents, if there was one. A statistical difference was considered to be \( p < 0.05 \).
Results and Discussion

Effect of PEG Grafting Degree on Contrast Enhancement

Both GDCP and PEGylated GDCP (0.03 mmol-Gd/kg) gave more prominent intravascular contrast enhancement in mice compared to a low molecular weight contrast agent, Gd(DTPA-BMA), at a dose of 0.1 mmol-Gd/kg. Coronal MR images of the heart of mice contrast enhanced with PEG2000H-GDCP, PEG2000L-GDCP, GDCP and Gd-(DTPA-BMA) at various time points are shown in Figure 20. Strong contrast enhancement was observed in the heart at 1 min postinjection with both PEG-GDCP polymeric contrast agents and GDCP at a dose of 0.03 mmol-Gd/kg compared to the enhancement from Gd-(DTPA-BMA) at a dose of 0.1 mmol-Gd/kg. The signal from GDCP decreased slightly after 15 min (Figure 20, column d), whereas the signal in the heart remained more intense over this period for each of the PEGylated agents, especially PEG2000H-GDCP. Compared to Gd-(DTPA-BMA), significant contrast enhancement was still visible in the heart at 60 min for the polymeric agents. The low molecular weight control agent, Gd-(DTPA-BMA), produced visible contrast enhancement at a dose of 0.1 mmol/kg 1 min postinjection and little to no enhancement afterward.

The ability of PEG-GDCP and GDCP macromolecular agents to enhance small vasculature in mice with excellent resolution is best demonstrated in Figure 21, which shows the coronal images of the descending aorta and common iliac arteries at various time points over 1 h. Although the contrast enhancement follows the same dynamics as in the heart, the macromolecular agents clearly revealed the blood vessels
Figure 20. Coronal MR images of mouse heart preinjection (a) and 1 (b), 5 (c), 15 (d), 30 (e) and 60 min (f) post intravenous injection of PEG$_{2000L}$-GDCP (A), PEG$_{2000H}$-GDCP (B), GDCP (C) and DTPA-BMA (D). Polymeric agents were given at a dose of 0.03 mmol-Gd/kg and Gd(DTPA-BMA) was given at the standard clinical dose of 0.1 mmol/kg.
Figure 21. Coronal MR images of descending aorta (→) and common iliac arteries (⇒) of the mouse preinjection (a) and 1 (b), 5 (c), 15 (d), 30 (e) and 60 min (f) post intravenous injection of PEG\textsubscript{2000}\textsubscript{L}-GDCP (A), PEG\textsubscript{2000}\textsubscript{H}-GDCP (B), GDCP (C) and Gd-(DTPA-BMA) (D). Polymeric agents were given at a dose of 0.03 mmol-Gd/kg and DTPA-BMA was given at the standard clinical dose of 0.1 mmol/kg.
1 min postcontrast while little contrast enhancement was observed with the control agent. The blood vessels with PEG_{2000H}-GDCP and PEG_{2000L}-GDCP agents were more visible through the first 5 min postinjection than compared with the images from un-PEGylated, GDCP. The smaller size of the GDCP (10.1 kDa) may have contributed to rapid renal filtration, whereas the clearance from PEG-GDCP (22.9 to 24.5 kDa) may have been more restricted.

PEGylation contributes to increased vascular retention (4), possibly by increasing the size of the agent or decreasing accessibility of the disulfide bond. The PEGylated macromolecular contrast agent demonstrated superior contrast enhancement in the heart and blood vessels as compared to the low molecular weight agent, Gd-(DTPA-BMA). Therefore, PEG-GDCP shows promise as a blood pool MRI contrast agent because of its increased signal intensity and retention time as well as its controllable degradation characteristics. Based on the enhancement characteristic in the heart, descending aorta, and common iliac arteries, the higher grafting of PEG-GDCP appears to be more favorable for imaging of the blood pool. This lead to the selection of high grafting ratio PEG for the next series of experiments on PEG-GDCP.

Effect of PEG Chain Length on Contrast Enhancement

Based upon visual evaluation, modifying the grafting ratios of biodegradable macromolecular GDCP with PEG-2000 significantly affected in vivo blood pool contrast enhancement of the contrast agents. PEG_{2000H}-GDCP with a high PEG grafting ratio (PEG/Gd = 0.76) provided more significant and prolonged contrast enhancement than either GDCP or PEG_{2000L}-GDCP (PEG/Gd = 0.33) in mice. A high grafting ratio of PEG-GDCP was therefore selected for further research. To further refine the effect of
PEG grafting for optimal contrast-enhanced MRI characteristics (maximal contrast enhancement for the length of a longer scan, but minimal tissue accumulation), the length of the PEG chains on the graft were varied. The second series of PEG-GDCP contrast agents were characterized by in vivo contrast enhancement.

The 3D maximum intensity projection (MIP) MR images of mice contrast enhanced with PEG\textsubscript{2000}-GDCP, PEG\textsubscript{1000}-GDCP, and PEG\textsubscript{550}-GDCP at various time points are shown in Figure 22. Gd-(DTPA-BMA) was not included with this group of contrast agents since PEG-GDCP was shown to have superior contrast enhancement in the first series of in vivo studies. In these images contrast enhancement of the heart, kidneys, vessels of the head and neck are all clearly shown through the first 5 min postinjection. The signal intensity then gradually dropped off based on the chain length of PEG in the graft copolymers. The contrast enhancement in the heart from PEG\textsubscript{2000}-GDCP remained strong throughout the first 15 min and was still visible at 30 min and had an observable signal at 60 min. PEG\textsubscript{1000}-GDCP gave enhancement profiles in which the signal intensity in the heart visibly decreased between 15 and 30 min and was near background levels at 60 min. The enhancement from PEG\textsubscript{550}-GDCP decreased more rapidly compared to PEG\textsubscript{1000}-GDCP, and it disappeared to background level after 30 min.

Similar contrast enhancement patterns were also observed in the finer blood vessels for three agents. Coronal slice images through the mouse abdomen shown in Figure 23 identify the descending aorta and common iliac arteries contrast enhanced by the agents. PEG\textsubscript{2000}-GDCP shows prominent enhancement of the vessels until one hour, while PEG\textsubscript{1000}-GDCP gives ample enhancement through 30 min and PEG\textsubscript{550}-GDCP through 15 min.
Figure 22. 3-D Maximum Intensity Projection (3D-MIP) MR Images of contrast enhancement of mice by PEG$_{2000}$-GDCP (A), PEG$_{1000}$-GDCP (B), and PEG$_{550}$-GDCP (C) at preinjection (a) and 2 (b), 5 (c), 10 (d), 15 (e), 30 (f), and 60 (g) min postinjection macromolecular contrast agent. Contrast agents were injected at a dose of 0.05 mmol-Gd/kg. The relatively high signal intensity in the gut is present in both pre- and postcontrast enhancement.
Figure 23. MR coronal slices through mouse blood vessels before (a) and 2 (b), 5 (c), 10 (d), 15 (e), 30 (f), and 60 (g) min after contrast enhancement by either PEG$_{2000}$-GDCP (A), PEG$_{1000}$-GDCP (B), or PEG$_{550}$-GDCP (C).
Figure 24 shows the ratios of the signal intensities of the heart, liver and kidney to muscle at various time points compared to the signal from muscle precontrast enhancement. The signal intensity ratio in the heart for PEG\textsubscript{2000}\textendash{}GDCP at 2 min was statistically different from the signal intensity in the same tissue compared to PEG\textsubscript{550}\textendash{}GDCP (p < 0.05) but not to PEG\textsubscript{1000}\textendash{}GDCP as determined by a one-way ANOVA statistical analysis with a Tukey posttest. PEG\textsubscript{2000}\textendash{}GDCP gave statistically different (p < 0.05) signal intensities in the heart compared to PEG\textsubscript{550}\textendash{}GDCP at all time points. This difference was most pronounced (p < 0.001) at 10, 15, and 30 min. The difference between PEG\textsubscript{1000}\textendash{}GDCP and PEG\textsubscript{2000}\textendash{}GDCP in the heart enhancement was statistically significant at 5 min after injection and thereafter. A statistical difference in the signal intensity between PEG\textsubscript{1000}\textendash{}GDCP and PEG\textsubscript{550}\textendash{}GDCP was not observed at any single time point in the heart, but the signal intensity ratio from PEG\textsubscript{1000}\textendash{}GDCP was always higher. The AUC from precontrast signal intensity ratio to the ratio at 60 min for each agent was also calculated and tested for statistical significance using a one-way ANOVA with a Tukey posttest. The AUC of PEG\textsubscript{2000}\textendash{}GDCP was significantly greater (p < 0.05) in the heart compared to either PEG\textsubscript{1000}\textendash{}GDCP or PEG\textsubscript{550}\textendash{}GDCP. The AUC was greater for PEG\textsubscript{1000}\textendash{}GDCP than PEG\textsubscript{550}\textendash{}GDCP, but the difference was not significant.

The size of PEG in PEG\textsubscript{n}\textendash{}GDCP significantly affects the contrast enhancement in the blood pool (Figures 22, 23 and 24A). Although MR signal intensity in the tissue of interest does not necessarily linearly correlate to the concentration of the contrast agents, the dynamic changes point to the impact of PEG size on the in vivo properties of the modified agents. PEG\textsubscript{2000}\textendash{}GDCP displayed more significant and persistent contrast
Figure 24. PEGₙ-GDCP contrast enhancement profile. Dynamic contrast enhancement profile of PEGₙ-GDCP contrast agents in the heart (A), kidney (B), liver (C), muscle (D). Signal Intensity Ratio is defined as the ratio of the signal intensity in the specific organ or tissue at each time point to the signal intensity from muscle precontrast enhancement. Values are shown as mean ± SD (N = 3).
enhancement in the blood pool than either PEG_{1000}-GDCP or PEG_{550}-GDCP, while the signal in the blood pool decays more quickly for PEG_{550}-GDCP than PEG_{2000}-GDCP and PEG_{1000}-GDCP. The phenomenon correlates well with the degradability of this series of PEG-GDCP contrast agents (Figure 17). The modification with PEG of high molecular weight results in contrast agents with a slower degradation rate and the most prolonged contrast enhancement in the blood pool. PEG increases the biocompatibility of biomedical polymers by sterically preventing interaction other biomolecules (3). Potentially, PEG-2000 creates a larger hydrodynamic volume than either PEG-1000 or PEG-550 around GDCP. Therefore, PEG_{2000}-GDCP has a larger PEG volume protecting the biodegradable GDCP backbone by decreasing the probability of a low molecular thiol or enzyme in the blood stream interacting with the disulfide bond.

The signal enhancement patterns in the kidneys were also clearly visible in the 3D MIP images (Figure 22). The kidneys were clearly identifiable at 2 min for each polymeric contrast agent (Figure 22, second column). For PEG_{2000}-GDCP this signal intensity was maintained out through 30 min and diminished at 60 min. The kidneys of mice given either PEG_{1000}-GDCP or PEG_{550}-GDCP were visible in the 3D-MIP images at 10 min, but not as intensely as with PEG_{2000}-GDCP after 2 min. After 10 min the kidneys were slightly visible for PEG_{1000}-GDCP until 30 min, but not for PEG_{550}-GDCP.

In the kidneys, the shape of the contrast enhancement curve was different compared to curve observed in the heart (Figure 24B). At 2 min postinjection of contrast agent, PEG_{1000}-GDCP showed a higher intensity in the kidneys. After 2 min, however, the signal intensity of both PEG_{1000}-GDCP and PEG_{550}-GDCP decreased more quickly than PEG_{2000}-GDCP, whose signal decayed much slower. Two minutes and thereafter,
PEG\textsubscript{2000}-GDCP had significantly higher signal intensity values compared to PEG\textsubscript{550}-GDCP. At 5 and 15 min postinjection, PEG\textsubscript{1000}-GDCP is significantly different from PEG\textsubscript{550}-GDCP as well, but by 60 min, the signal intensity ratios were similar and the curves were rapidly approaching each other. PEG\textsubscript{2000}-GDCP did not become significantly different from PEG\textsubscript{1000}-GDCP, most likely due to the relatively larger standard deviation in the enhancement of PEG\textsubscript{2000}-GDCP and PEG\textsubscript{1000}-GDCP in the kidney.

Similar to the heart, the signal decay of the contrast agents modified with high molecular weight PEG in the kidney is slower than those with a lower molecular weight PEG by observing the 3D MIP images (Figure 22). Comparing the signal intensity ratio curves, however, the shape of the curve is different (Figure 24B), where PEG\textsubscript{2000}-GDCP resulted in more persistent contrast enhancement in the kidney than PEG\textsubscript{1000}-GDCP and PEG\textsubscript{550}-GDCP. Two major factors, slower degradation rate of PEG\textsubscript{2000}-GDCP and the relatively larger size of PEG-2000, may contribute to the slow clearance of PEG\textsubscript{2000}-GDCP in the kidneys. MPEG (MW \textsubscript{PEG} = 2000) is less readily cleared than PEG chains of shorter length via glomerular filtration (3). Therefore, the prolonged blood pool and kidney enhancement of PEG\textsubscript{2000}-GDCP may be attributed to the slow degradation rate of the macromolecules (Figure 18) and more limited renal excretion of the degradation products due to the longer PEG chains.

In the highly vascularized liver, the contrast agents behaved similarly as in the heart, Figure 24C. PEG\textsubscript{2000}-GDCP always had the highest signal intensity and was significantly different than PEG\textsubscript{550}-GDCP at all time points. At 10 and 15 min PEG\textsubscript{1000}-GDCP showed a significantly different enhancement pattern from PEG\textsubscript{2000}-GDCP. Considering
the AUC for each of the agents, PEG\textsubscript{2000}-GDCP had a significantly greater value compared to both PEG\textsubscript{1000}-GDCP and PEG\textsubscript{550}-GDCP. Overall the enhancement from the three polymeric contrast agents in the liver was lower than in either the heart or the kidneys. Considering Figure 22, the initial signal intensity in the liver of each agent was lower than its signal intensities in the heart and kidney as visualized in the 3D-MIP images. The agent modified with high molecular weight PEG had relatively higher signal intensity in the liver, which is consistent with the observations from the heart and kidney. Figure 24D shows that there was little contrast enhancement in the muscle and the difference between the agents was not significant. This finding supports that the contrast agents were limited to the blood pool before renal clearance.

PEG is often used to modify proteins and polymers to alter their pharmacokinetics and biodistribution. It has been shown that the presence of PEG on proteins decreases their uptake by reticuloendothelial cells while obtaining a longer half-life (4). PEG grafting has also been used on dendrimeric contrast agents, resulting in decreased liver uptake and more prolonged plasma enhancement (5). A similar effect was seen with the grafting of PEG to Gd(DTPA)-polylysine (6). It was expected that the modification of the biodegradable macromolecular MRI contrast agents with PEG improves their pharmacokinetic properties and minimizes nonspecific tissue uptake, particularly the uptake in the liver.

In the first two series of contrast agents, the apparent molecular weight of GDCP was increased for the second series compared to the first. This property was expected to be minimal. Comparing GDCP grafted with PEG-2000 at a high ratio for the first and second series of PEG-GDCP contrast agents, both contrast agents showed similar contrast
enhancement patterns as shown in Figure 20 (row B) and Figure 22 (row A). These dynamic contrast enhancement series demonstrate that the overall apparent molecular weight contributes more significantly to blood pool contrast enhancement than the backbone GDCP molecular weight.

The study investigated the effect of PEG of different sizes on the physicochemical properties and in vivo contrast enhancement of PEGylated biodegradable macromolecular contrast agents. It was demonstrated that these properties are significantly altered by the size of the PEG. PEG$_{2000}$-GDCP showed the most prominent and prolonged contrast enhancement in the blood pool, while PEG$_{550}$-GDCP and PEG$_{1000}$-GDCP were cleared more quickly. The longer PEG grafts resulted in more persistent contrast enhancement in the kidney, indicating that the length of PEG may affect renal clearance of the agents. It may be hypothesized that PEG-2000 has some limitation to glomerular filtration, whereas PEG-1000 and PEG-550 do not and therefore degradation products from PEG$_{1000}$- or PEG$_{550}$-GDCP may be readily filtered, while PEG$_{2000}$-GDCP may have prolonged retention.

**Summary**

Conjugation of PEG-2000 at varying grafting ratios in the first series of agents resulted in contrast agents that showed appreciably higher contrast enhancement in the blood pool compared to a control agent. PEG$_{2000H}$-GDCP, which had the higher grafting degree of the two PEG-GDCP agents tested, showed more prominent enhancement in the blood pool, represented by enhancement in the heart, and better delineation of the small vessels as observed in the descending aorta and common iliac arteries. These results
suggested that PEG-GDCP of a higher grafting degree should be investigated for next series of PEG-GDCP contrast agents.

In the second series of contrast enhanced MR exams with PEG-GDCP, a higher PEG grafting degree was selected and the size of the PEG chain was manipulated to further define an optimal PEG-GDCP contrast agent. PEG\textsubscript{2000}-GDCP showed enhancement in MIP images out to the longest time point (Figure 22), but in coronal cross-sections of the small vasculature in mice PEG\textsubscript{1000}-GDCP showed prominent enhancement out to 30 min as well (Figure 23). To be an effective blood pool contrast agent, the potential gain in enhancement by the agent must be weighed against the potential toxicity from prolonged circulation. Figure 24B shows the dynamic enhancement curve of PEG\textsubscript{r}-GDCP in the kidney. The more sustained contrast enhancement and different curve shape from PEG\textsubscript{2000}-GDCP in the kidney indicate that its clearance is possibly restricted via renal filtration, which may result in long-term Gd(III) accumulation. Compared to PEG\textsubscript{2000}-GDCP, the clearance of PEG\textsubscript{1000}-GDCP did not appear to be restricted. PEG\textsubscript{1000}-GDCP also showed considerable enhancement of the blood pool comparable to PEG\textsubscript{2000}-GDCP and clearly better than PEG\textsubscript{550}-GDCP (Figure 23). Therefore, to combine the best possible contrast enhancement, yet minimize the potential of long-term Gd(III) accumulation, PEG\textsubscript{1000}-GDCP was the selected most promising candidate for future study.

**Literature Cited**


CHAPTER 6

PHARMACOKINETIC AND LONG-TERM GADOLINIUM(III) RETENTION STUDIES WITH GDCP AND PEG-GDCP

Macromolecular polydisulfide Gd(III) complexes initially circulate in the blood pool as a macromolecular contrast agent. Over a reasonable period of time, the polymer gradually breaks down via the thiol–disulfide exchange reaction into low molecular weight Gd(III) monomers and oligomers that are efficiently eliminated via renal filtration (1). For example, GdDTPA-cystamine copolymers (GDCC), GdDTPA-cystine copolymer (GDCP), GdDTPA-cystine ethyl ester copolymers (GDCEP) all show superior contrast enhancement in the blood pool for a longer period of time compared to a clinically approved low molecular weight contrast agent, Gd(DTPA-BMA). Yet, based on the chemical structure around the disulfide bond these agents were eliminated from the blood stream at different rates with minimal long-term Gd(III) accumulation (2-5). These studies demonstrated that the pharmacokinetics and Gd(III) retention are altered by the chemical structure around the disulfide bond. Appropriate modification is possible and it is critical to optimize the blood pool retention of these agents while minimizing long-term Gd(III) retention.

In the two previous studies (see Chapters 3-5), varying the degree of PEG grafting (6) and PEG chain length (7) to GDCP resulted in a variety of physiochemical properties and dynamic enhancement characteristics that showed potential for future investigation.
Poly(ethylene glycol) conjugation was used in this body of work to modify the chemical structure around the disulfide bond. PEG grafting is routinely used to increase biocompatibility, modify plasma circulation, and alter long-term biodistribution of macromolecules (8). Little is known on the plasma pharmacokinetics, long-term Gd(III) accumulation, and in vivo metabolism of the PEGylated polydisulfide agents. A lead agent, PEG1000-GDCP (PEG, MW = 1000 Da), was selected from these previous studies. This agent was selected for in vivo Gd(III) plasma pharmacokinetics, biodistribution, and metabolic analysis because the contrast enhancement was sufficient for at least 15 to 30 min in rats. Unlike PEG2000-GDCP, however, the renal clearance of PEG1000-GDCP does not seem to be restricted at longer periods of time. Thus, PEG1000-GDCP appears to have the proper balance between imaging efficacy and minimal long-term Gd(III) accumulation. An optimal pharmacokinetic and biodistribution profile would indicate its potential application for future development as a magnetic resonance angiography (MRA) contrast agent.

Methods

Physiochemical parameters of the contrast agents used in these experiments are shown in Table 2 and discussed in Chapter 3. Briefly, PEG1000-GDCP of three different PEG grafting degrees were synthesized and characterized [see Chapter 3, poly(GdDTPA-co-L-cystine) – method three and PEG1000x-g-poly(GdDTPA-co-L-cystine)]. The molecular weight for PEG1000H-GDCP, PEG1000M-GDCP, PEG1000L-GDCP, and GDCP were 42.1, 46.5, 52.1, and 43.3 kDa, respectively. Figure 25 shows the size exclusion chromatography (SEC) profiles for these agents. The profiles significantly overlap indicating that all agents used in these studies had similar hydrodynamic size.
Figure 25. Size exclusion chromatography (SEC) profiles of the biodegradable polydisulfide Gd(III) complexes used in the pharmacokinetic, long-term Gd(III) retention, and in vivo metabolism studies. Contrast agent properties are given in Table 2, page 71.

The PEG grafting degree was 0.96, 0.65, and 0.39 PEG/Gd for the high, medium, and low for PEG1000x-GDCP, respectively. The relaxivity for all four agents ranged from 5.0-6.1 mM⁻¹s⁻¹. All animal experiments reported in this chapter were performed under an approved protocol from the University of Utah Institutional Animal Care and Use Committee.

Pharmacokinetics

A pharmacokinetic study of biodegradable macromolecular contrast agents was tested in male Sprague-Dawley rats (170-230 g; Charles River Laboratories, Wilmington, MA). Each agent was tested in a group of 6 rats. The rats were first sedated by
isoflurane gas and then fully anesthetized by the intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). A heparinized catheter was then inserted into the jugular vein of the rat for contrast agent administration and blood sampling. All contrast agents were injected at a dose of 0.1 mmol-Gd/kg followed by a saline push (250 μl) to rid the catheter line of residual contrast agent. Blood samples (150 – 200 μl aliquots) were taken before the injection of contrast agent and at 2, 4, 6, 8, 10, 15, 30, 60, 120, 180, and 360 min postinjection of contrast agent. After each blood sample was taken, the catheter was washed with an equal volume of heparinized saline to maintain fluid volume in the rat and to avoid subsequent sample contamination. The blood samples were immediately centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant plasma was diluted with Milli-Q® water (Millipore Corporation, Bedford, MA) and the Gd(III) concentration were determined by ICP-OES.

Data from the pharmacokinetic experiment were analyzed with a standard open two-compartment model for an intravenous bolus input using WinNonlin® (Pharsight Corporation, Mountain View, CA). The distribution and elimination phase half-lives, $t_{1/2, α}$ and $t_{1/2, β}$, respectively, and the initial distribution volume into the central compartment ($V_c$) were calculated based on the results of the two-compartment model. In addition, the 10 and 30 min AUC values for each contrast agent were calculated. Statistical calculations were performed with GraphPad Prism (GraphPad Software, San Diego, CA) software. For each group of four contrast agents, each pharmacokinetic time point, the half-life parameter values, and the Gd(III) retention for each organ or tissue were all analyzed with a one-way ANOVA with Bonferroni’s Multiple Comparison Test. Statistical significance was considered to be $p < 0.05$. 
Long-Term Gd(III) Retention

The long-term tissue accumulation of Gd(III) was tested in a group of 6 male Sprague-Dawley rats for each contrast agent. Contrast agents were injected intravenously via a tail vein at a dose of 0.1 mmol-Gd/kg and placed in metabolic cages. Urine samples were collected for the first 24 h to identify potential Gd(III) metabolites and to verify renal clearance of the agents, measured by ICP-OES. At 10 days postinjection of contrast agent, the rats were sacrificed with an overdose of isoflurane. Organ or tissue samples from the heart, kidney, liver, lung, muscle, spleen, and femur were collected and weighed. All organs were dissolved in 1.0 ml of 70% HNO₃ (Nitric Acid Optima, Fisher Chemical) and within two days the tissues were liquefied. The samples were then centrifuged at 4000 rpm for 20 min at 4°C. The supernatant was diluted five times in Milli-Q® water and further centrifuged at 10,000 rpm for 15 min. The supernatant was collected and Gd(III) contents were determined by ICP-OES. The weight of the heart, kidney, liver, lung, femur, spleen, and muscle were estimated to be 0.447%, 0.88%, 4.064%, 0.661%, 0.4%, 0.267%, and 40%, respectively, of the total weight of the rat (2).

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Urine samples that were collected during the first eight hours postinjection of each contrast agent were subjected to a metabolic analysis using both positive- and negative-charge-labeled MALDI-TOF mass spectroscopy on an α-cyano-4-hydroxycinnamic acid matrix. Metabolite Gd(III) complexes were identified based on characteristic Gd(III) isotope mass distribution patterns.

Following the Gd(III) tissue retention experiment, each rat (including 3 control rats that were administered saline) was euthanized with an isoflurane overdose. A left
nephrectomy immediately following sacrifice was performed to look for signs of potential renal toxicity. Full mount 5 mm thick sections were obtained from each nephrectomy specimen and were fixed in a 10% neutral buffered formalin solution (Mallinckrodt, St. Louis, MO). Each kidney was then processed by a conventional tissue processing method (9) and two 5 μm sections were prepared by routine hematoxylin and eosin (H&E) staining for histological analysis. A pathologist (L.L.E.) blinded to the results of the pharmacokinetic, tissue retention, and MRI experiments, examined each section for evidence of tubular injury. The degree of tubular injury was assessed using a semi-quantitative method. Five random high power fields (40X) of the renal cortices were reviewed for each kidney, and an average histologic score for each kidney was generated based on the following scoring method: 0 – no significant tubular injury; 1 – tubular cell swelling, loss of brush border, nuclear condensation (apoptosis), and up to 1/3 of the tubular cross sections showing nuclear loss (necrosis); 2 – same as 1, except for > 1/3 but < 2/3 nuclear loss in tubular cross sections; 3 – same as 2, except for > 2/3 nuclear loss in tubular cross sections.

Results
Pharmacokinetics

Figure 26 shows the blood plasma Gd(III) concentration versus time profile for PEG_{1000H}-GDCP, PEG_{1000M}-GDCP, and PEG_{1000L}-GDCP, and GDCP for the first 6 h postintravenous injection of contrast agent in rats. At 2 min postinjection of the agents, all three PEG-GDCP (398-454 mg/L) contrast agents had a higher blood plasma concentration than GDCP (274 mg/L) of similar molecular weight, resulting in a lower distribution volume for the three PEGylated agents (Table 3). Although the difference
Figure 26. Blood clearance of Gd(III) complexes in rats after intravenous injection of GDCP and PEG$_{1000}$X-GDCP. Each contrast agent was injected at a dose of 0.1 mmol-Gd/kg. Values are shown as the mean ± SD, $N = 6$. The inset shows plasma Gd(III) concentration for the first 30 min on a linear-linear scale; error bars are not shown for clarity.
Table 3
Pharmacokinetic Parameters of GDCP and PEG1000x-GDCP

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<thead>
<tr>
<th></th>
<th>PEG1000H-GDCP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PEG1000M-GDCP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PEG1000L-GDCP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GDCP&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>AUC&lt;sub&gt;10&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (mg/L*min)</td>
<td>1348.7 ± 269.5</td>
<td>1645.0 ± 383.6</td>
<td>1255.5 ± 307.0</td>
<td>1083.9 ± 84.9</td>
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<td>AUC&lt;sub&gt;30&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; (mg/L*min)</td>
<td>2467.7 ± 365.7</td>
<td>2789.8 ± 735.6</td>
<td>2233.7 ± 850.1</td>
<td>2203.2 ± 267.6</td>
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<tr>
<td>V&lt;sub&gt;c&lt;/sub&gt; (ml)</td>
<td>2.8 ± .4</td>
<td>1.8 ± .5</td>
<td>2.6 ± .9</td>
<td>7.1 ± .4</td>
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<tr>
<td>t&lt;sub&gt;1/2, α&lt;/sub&gt; (min)</td>
<td>0.87 ± 0.67</td>
<td>0.49 ± 0.27</td>
<td>0.55 ± 0.17</td>
<td>4.14 ± 3.88</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2, β&lt;/sub&gt; (min)</td>
<td>11.3 ± 2.2</td>
<td>7.4 ± 0.5</td>
<td>6.5 ± 1.5</td>
<td>196.7 ± 107.5</td>
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<sup>a</sup>Contrast agents were administered intravenously via a jugular vein at dose of 0.1 mmol-Gd/kg in rats. Values are shown as the mean ± SD, N = 6.

<sup>b</sup>Area under the curve after 10 min (AUC<sub>10</sub>) postinjection of contrast agent.

<sup>c</sup>Area under the curve after 30 min (AUC<sub>30</sub>) postinjection of contrast agent.
was not significant at any single time point, PEG-GDCP consistently had a higher Gd(III) plasma concentration for the first 10 min postinjection, which is best represented by looking at the AUC$_{10}$ and AUC$_{30}$ (Table 3). By 15 min postinjection, all four contrast agents had similar Gd(III) complex concentration in the blood plasma. From 15 min through 6 h postinjection, however, the Gd(III) complex plasma concentration from each of the three PEG-GDCP contrast agents dramatically decreased compared to GDCP. The difference in plasma concentration of GDCP compared to PEG$_{1000M}$-GDCP and PEG$_{1000L}$-GDCP was significant from 30 min until the end of the experiment, according to Bonferroni’s multiple comparison posttest. The difference in plasma concentration between GDCP and PEG$_{1000H}$-GDCP did not become statistically significant ($p < 0.001$) until 60 min postinjection.

A two-compartment pharmacokinetic model was implemented to analyze the Gd(III) plasma concentration profiles. Table 3 lists the results for the distribution ($\alpha$) and elimination ($\beta$) phase half-lives for each of the four biodegradable macromolecular Gd(III) complexes tested. A one-way ANOVA test was used to test for statistical significance for each group of contrast agents, while Bonferroni’s posttest was used to determine significant differences between pairs of data. The elimination half-life of GDCP (196.7 min) was significantly larger ($p < 0.001$) than any of the PEG-GDCP contrast agents, counterintuitive to prior expectation that PEG grafting increases the circulation half-life. Among the PEG-GDCP contrast agents, PEG$_{1000H}$-GDCP had the longest elimination half-life (11.3 min), while PEG$_{1000M}$-GDCP and PEG$_{1000L}$-GDCP had shorter elimination half-lives, 7.4 and 6.5 min, respectively, depending on the degree of PEG grafting, but these differences were not significant. Examining the distribution half-
life, the value of GDCP was the largest (4.14 min), while the value for each of the PEG-GDCP contrast agents was less than 1 min. The difference was significant \( (p < 0.05) \) for both PEG\(_{1000M}\)-GDCP and PEG\(_{1000L}\)-GDCP compared to GDCP, but not PEG\(_{1000H}\)-GDCP. Initially, the pharmacokinetics for biodegradable contrast agents PEG-GDCP and GDCP were similar; however, after 30 min, the clearance of PEG-GDCP becomes significantly more rapid.

**Long-term Gd(III) Retention**

The long-term (10 days) tissue accumulation of PEG\(_{1000H}\)-GDCP, PEG\(_{1000M}\)-GDCP, PEG\(_{1000L}\)-GDCP, and GDCP in the heart, kidney, liver, lung, femur, spleen, and muscle after a dose of 0.1 mmol-Gd/kg is shown in Figure 27. Except for the kidney and muscle, the tissue retention of GDCP was significantly higher \( (p < 0.01) \) than any of the PEG-GDCP contrast agents. The higher accumulation of GDCP in the liver was most obvious compared to PEG-GDCP where there was not a significant difference between any pair of PEG-GDCP agents. GDCP also had significantly higher accumulation in the femur and spleen. In the kidney, PEG\(_{1000H}\)-GDCP and PEG\(_{1000M}\)-GDCP had significantly lower Gd(III) accumulation than GDCP. The Gd(III) accumulation in kidney was lower for PEG\(_{1000L}\)-GDCP compared to GDCP, but the difference was not significant. In the muscle, PEG\(_{1000H}\)-GDCP had a significantly lower Gd(III) accumulation than PEG\(_{1000M}\)-GDCP, PEG\(_{1000L}\)-GDCP, and GDCP. Overall, PEG-GDCP had a substantially lower Gd(III) tissue accumulation in every organ or tissue except muscle, where PEG\(_{1000M}\)-GDCP and PEG\(_{1000L}\)-GDCP had slightly higher accumulations.
Figure 27. Biodistribution of Gd(III) in rats 10 days after the intravenous injection of GDCP and PEG_{1000X}-GDCP. Each contrast agent was injected at a dose of 0.1 mmol-Gd/kg. Values are shown as the mean ± SD, N = 6. *p < 0.05 for GDCP compared to all three PEG_{1000X}-GDCP contrast agents according to Bonferroni’s Multiple Comparison Test.
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For each agent, the majority of Gd(III) was recovered in the urine after 24 h. Figures 28 and 29 show the positive- and negative-charge-labeled MALDI-TOF mass spectroscopy urine metabolite analysis. The spectra are from the cumulative urine collected after 8 h from each agent. For both positive- and negative-charge-labeled MALDI-TOF MS, similar Gd isotope mass distributions were present indicative of the degradation of the polydisulfide contrast agent into small Gd(III) chelates. Major metabolites for PEG1000H-GDCP had a mass (m/z) of approximately 877.0, 971.0, and 988.0 in the positive-charge-labeled mass spectrum of the urine sample (Figure 28A). Minor peaks were present at 895.0, 1176.0, and 1214.0. The positive-charge-labeled mass spectra of PEG1000M-GDCP and GDCP are similar to that of PEG1000H-GDCP, while the mass spectrum of PEG1000L-GDCP had significantly smaller minor peaks and only one major peak at 988.0 (Figure 28B-D).

A Gd(III) isotope pattern was observed as the major peak at 988. This peak has been observed in the MALDI-TOF in urine after the injection of polydisulfide Gd(III) complexes, but the peak has yet to be resolved (5,10). The peak around 971, also has a Gd isotope pattern. The mass of 971 may correspond to single contrast agent repeat unit that was decarboxylated at both possible positions on l-cystine as well as having one of the thiols of the degradation unit oxidized with the thiol group of glutathione. The acidic the matrix used for MALDI-TOF would allow this molecule to be observed in the positive-charge-labeled mass spectrum since the tripeptide glutathione has a primary amine.
Figure 28. Positive-charge-labeled MALDI-TOF MS from the urine of rats administered PEG<sub>1000H</sub>-GDCP (A), PEG<sub>1000M</sub>-GDCP (B), PEG<sub>1000L</sub>-GDCP (C), and GDCP (D). Indicated peaks have Gd isotope peaks.
Figure 29. Negative-charge-labeled MALDI-TOF MS from the urine of rats administered \( \text{PEG}_{1000} \text{H}-\text{GDCP} \) (A), \( \text{PEG}_{1000} \text{M}-\text{GDCP} \) (B), \( \text{PEG}_{1000} \text{L}-\text{GDCP} \) (C), and GDCP (D). Indicated peaks have Gd isotope peaks.
Figure 29 shows that the negative-charge-labeled mass spectrum is relatively more complicated than the positive-charge-labeled spectrum. At 8 h postinjection of contrast agent, the mass spectrum was similar for each of the four urine samples. In the negative-charge-labeled mass spectrum, major metabolites that demonstrated Gd(III) isotope mass distribution were at 588.2-589.2 and at 910.1-910.2. Smaller peaks were also present at around 636, 659, 696, and 734. The peaks identified between 589 and 734 were broad revealing a mass distribution more complicated than monomeric Gd(III) degradation units. The chemical structures of most of these metabolites remain unknown because of further metabolism of the degradation products. The peaks at 589, 734, and 910 are consistent with metabolite molecular weights previously reported with polydisulfides (5). These results demonstrate that PEG grafts are potentially hydrolyzed from the polymeric contrast agents. In vivo, the metabolic profile is quite different compared to the in vitro degradation assay supporting that the in vivo environment is more complicated than just the thiol-disulfide exchange reaction. Further studies are on going to better elucidate the in vivo mechanism of degradation and the exact catabolite structure.

The histopathologic results show minimal toxicity of PEG1000X-GDCP and GDCP when compared to a saline injection of equivalent volume. For each contrast agent 30 random high power fields (HPF) (6 kidney specimens per agent with 5 HPFs observed per kidney) at 40X were observed. Injection of PEG1000H-GDCP resulted in 29 of 30 (96.7%) HPFs having a score of 1, indicating mild tubular damage. A score of 1 was observed in 15 of 15 (100%) of saline controls (3 kidney specimens). A score of 2, indicating moderate tubular damage, was observed in only 1 of 30 HPFs (3.3%) for PEG1000H-GDCP and PEG1000L-GDCP and 3 of 30 HPFs (10%) for PEG1000M-GDCP.
There were no HPFs with a score of 2 for GDCP. No significant tubular damage (score 0) was identified in 1 of 30, 3 of 30, and 2 of 30 HPFs for PEG\textsubscript{1000M}-GDCP, PEG\textsubscript{1000L}-GDCP, and GDCP, respectively. Severe tubular damage was not seen with any of the agents.

Using a nonparametric one-way ANOVA statistical test (Kruskal-Wallis) for significance with Dunn’s posttest, there was no statistical difference between any of the contrast agents or with any individual contrast agent and a saline control. The findings for the renal toxicity experiment are summarized in Table 4. 109 of 120 (90.8%) of all HPFs from the experimental agents and 15 of 15 (100%) of the saline controls received a score of 1 (Figure 30). No significant histopathologic changes were identified in the interstitial, glomerular, or vascular compartments. A small focal medullary infarct was noted in one rat tested with PEG\textsubscript{1000M}-GDCP. A score of 1, even in the control rats, was most likely observed because the isoflurane overdose used for euthanasia potentially resulted in an immediate premortem hypoxic event. The absence of an interstitial inflammatory response also supports this score representing an acute hypoxic event rather than toxicity resulting from contrast agent (11).

**Discussion**

Initially the three PEG-GDCP contrast agents have a higher plasma concentration than GDCP leading to decreased distribution volumes and higher values for the AUC at both 10 and 30 min postinjection for all PEG-GDCP contrast agents (Table 3). It was previously shown that the degradation of the disulfide containing biodegradable macromolecular contrast agents possibly starts immediately after injection (2,3). The
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<th>Histologic Score&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>2</td>
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<tr>
<td><strong>Saline&lt;sup&gt;c&lt;/sup&gt;</strong></td>
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<sup>a</sup>0 – no significant tubular damage; 1 – mild tubular damage; 2 – moderate tubular damage; 3 – severe tubular damage. Please see accompanying text for further explanation.

<sup>b</sup>6 rat kidney specimens × 5 random high power fields (40X) = 30 total measurements per agent.

<sup>c</sup>3 rat kidney specimens × 5 random high power fields (40X) = 15 total measurements per agent.
Figure 30. Representative renal histopathologic images of GDCP (A) and a saline control (B) 10 days after the intravenous injection of the respective compound. These images each a score of 1 indicating mild tubular damage; H&E, 60X.
increased initial concentration of PEG-GDCP suggests that PEGylation of GDCP may delay the onset of degradation or that early degradation products are large, most likely because the hydrodynamic effects of PEG grafting. After 15 min, however, the plasma clearance of the Gd(III) complexes from PEG-GDCP is greatly increased compared to GDCP (Figure 26). Modification of a polyanion with PEG reduces the overall negative charge of the contrast agent and therefore likely modifies the plasma stability of the contrast agent. Zong et al. recently reported that modification polyanionic GDCP by ethylation of the carboxylate groups of L-cystine (GDCEP) greatly reduced the stability of disulfide bond in rat plasma (4). In another study, modification of cystine in GDCP to a bisamide or a bisalkylamide greatly increased the rate of in vitro degradation to a thiol, L-cysteine (5). Electrostatic repulsion between GDCP and thiolate anions in the plasma most likely accounts for the decreased degradation rate of GDCP (12,13). The pharmacokinetic results from PEG-GDCP indicate that charge may have a governing role in the degradation of the agents in vivo, such that reduction or elimination of the negative charge surrounding the disulfide bond greatly reduces its stability. Further studies are required to better understand the mechanism of in vivo polydisulfide degradation.

The significantly longer terminal half-life of GDCP compared to PEG-GDCP is evident when examining the long-term Gd(III) retention. The accumulation of Gd(III) complexes from GDCP is significantly higher in every tissue except the kidney and muscle. The most considerable difference between PEG-GDCP and GDCP occurs in the liver ($p < 0.001$). Several factors likely contribute to the significantly higher accumulation of GDCP in the liver compared to PEG-GDCP. The first major factor is that the terminal elimination half-life of GDCP is 17-30 times longer than any of the
PEG-GDCP contrast agents. The other major factor is related to the charge of GDCP. Macrophage scavenger receptors have a broad binding specificity to foreign bodies that are polyanionic (14). It has been shown with several model biomacromolecules that increasing the overall negative charge increases uptake of the negatively charged molecules by sinusoidal cells of the liver, specifically endothelial and Kupffer cells (15,16). On the other hand, PEGylation of biomolecules has been shown to decrease uptake of reticuloendothelial cells by masking both charges and recognition sites (8). Decreased hepatic uptake was also observed for both PEGylated dendrimeric (17) and PEGylated linear polymeric contrast agents (18).

In Chapter 4, it was shown that the degradation products in vitro had intact PEG conjugation. In vivo, however, there were no degradation units in the first eight hours that had a molecular weight high enough to have intact PEG. There may be some polycharged oligomers where PEG was still conjugated, but the degradation spectrum from a polycharged, poly dispersed oligomer with a polydisperse graft would be too complex to interpret and confirm the structure from a urine sample. We can assume that because the clearance rate profile shows rapid clearance, that if there were readily identifiable PEGylated subunits, they would be visible at 8 h. Total identification of all catabolites in vivo is difficult due to the potential of further metabolism and alternative degradation pathways not encountered in vitro. For example, it was recently reported that cysteine dioxygenase (CDO) acts quite indiscriminately in metabolizing thiols (19,20). The enzyme has been reported to be present in both the liver and the renal cortical tubules of the kidney (21). Although this enzyme may or may not be responsible for initial disulfide reduction, it may further modify a thiol into a sulfinic acid. MALDI-
TOF mass spectroscopy results of the urine suggest enzymatic metabolism is a possibility due to the larger number of unidentifiable peaks and hydrolysis of PEG chains since the degradation units of all four contrast agents were similar. As expected degradation of polydisulfides in vivo is complex more involved than just reduction of disulfides.

Here we show that PEG-GDCP gave higher initial plasma concentrations of Gd(III) than GDCP, but had significantly lower long-term Gd(III) accumulation. These studies demonstrate that PEG-GDCP displays optimal plasma retention, long enough for high-resolution MRI pulse sequences of the blood pool, but with minimal Gd(III) accumulation. Further investigation is needed to better understand the degradation mechanisms and metabolism of polydisulfides. These current results confirm that Gd(III) ions are being excreted as intact low molecular weight chelates.

**Summary**

PEG-GDCP yields higher Gd(III) plasma concentration than GDCP in the first 15 min postinjection. Elimination of PEG-GDCP from blood pool was dramatically increased compared to GDCP after a half hour. The plasma concentration profile of PEG-GDCP should be ideal for longer high-resolution contrast enhanced MRI pulse sequences because of its higher concentration immediately after injection and through 15 min. In addition, PEG-GDCP has significantly lower Gd(III) accumulation at 10 days in all major organs compared to GDCP. The difference was greatest in the liver. At 8 h postinjection, low molecular weight Gd(III) chelates were present in the urine. The mass spectra suggested both further metabolism of the degradation units and elimination of PEG from the GDCP backbone. The combination of a majority of Gd(III) metabolites in
urine, minimal renal toxicity, and minimal liver accumulation support safe renal excretion of PEG-GDCP.

**Literature Cited**


MAGNETIC RESONANCE ANGIOGRAPHY
WITH GDCP AND PEG-GDCP

Magnetic resonance angiography (MRA) is a diagnostic application of MRI that is used to evaluate arterial and venous blood flow. Specifically, time of flight (TOF)-MRA gives high amplitude signal to laminar flowing blood by saturating the signal from stationary tissue, such as muscle. In many cases, such as patients with aneurismal disease, poor cardiac output, or tortuous vessels, examining only the blood flow is not sufficient. Therefore, contrast-enhanced MRA (CE-MRA), where a contrast agent is used to significantly decrease the $T_1$ of blood compared to that of surrounding tissue, is used for the majority of clinical MRA exams. Low molecular weight paramagnetic Gd(III) chelates are routinely used in clinical situations to decrease the $T_1$ relaxation time of blood and consequently improve MR images ($J$). However, current clinically used MRI contrast agents are not well-suited for the needs of angiographic MR examinations. These contrast agents have very short plasma half-lives and readily extravasate across healthy vasculature endothelium into the surrounding tissue (2-4). As a result, the image resolution is compromised from dynamic Gd(III) plasma concentration (the exam is much longer than peak enhancement), $T_1$ shortening of surrounding tissue, and difficulty of exact timing between of the contrast agent bolus and image acquisition ($J$). In addition an increased dose up to three times more than the FDA approved dose of
contrast agent is needed to effectively decrease the $T_1$ relaxation time of blood in this type of imaging (1).

Macromolecular Gd(III) complexes for CE-MRA are a promising alternative to the inadequacy of current clinically approved contrast agents. Rigorous effort toward the design of macromolecular Gd(III) complex has emerged to accomplish the need of a contrast agent for angiographic MR protocols. Contrast agents based on synthetic polymers (6-8), natural macromolecules (9), and colloids (10) have been designed and are reviewed in Chapter 1. Most notably, macromolecular Gd(III) complexes increase plasma circulation time compared to low molecular weight contrast agents. In addition, since the larger size of macromolecules prevents their extravasation from healthy vasculature, their distribution is limited to the intravascular space of the vessel lumen (11,12). Macromolecular Gd(III) complexes also have increased $T_1$ relaxivity compared to low molecular weight contrast agents (13), which results in $T_1$ shortening that is limited to the blood pool and not surrounding tissue at a lower dose of Gd(III). Therefore, macromolecules are the ideal medium to achieve definitive high-resolution angiographic images.

To date, however, there are no macromolecular Gd(III) complexes approved for clinical use due to potential toxicity from slow elimination and high long-term Gd(III) accumulation. For example, polylysine-GdDTPA conjugate has up to a 428.6 min half-life in rats (14), while Gd(III) complexes to carboxymethyl hydroxyethyl starch and second generation polypropyleneimine had 47% retention and 45% retention in rats after seven and fourteen days, respectively (15,16). To realize the full potential of
Polydisulfide biodegradable macromolecular Gd(III) complexes have shown great potential as contrast agents for blood pool MRI applications. In the previous several chapters, the design and development of poly(ethylene glycol) grafted polydisulfide Gd(III) complexes has been presented. Based on preliminary results on contrast enhancement dynamics in healthy mice, a lead agent PEG\textsubscript{1000}-GDCP was selected for further development for its promising pharmacokinetics, long-term Gd(III) retention, and in vivo metabolism (Chapter 6). It was hypothesized that this agent would have the optimal combination of prolonged blood pool circulation sufficient for the length of high-resolution scan, but it would still show minimal long-term Gd(III) accumulation. Results from that study indicated that at initial time points PEG-GDCP had a distribution more limited to the intravascular space. After a half-hour, however, the contrast agent was readily cleared with minimal long-term Gd(III) accumulation.

Concomitant with the animal tissue experiments in Chapter 6, the lead PEG-GDCP agent was also tested for its efficacy in an MR angiography protocol. One of the major limitations of MRA at this point is the high dose required from clinically approved low molecular weight contrast agents. The results of the pharmacokinetic and long-term Gd(III) retention experiment showed a favorable profile at a standard clinical dose of 0.1 mmol-Gd/kg for comparison with literature values. Previous experiments with PEG-GDCP and GDCP showed that effective contrast enhancement could be obtained at lower doses, however. Therefore PEG-GDCP and GDCP were tested at a dose of 0.03 mmol-Gd/kg. Even though the pharmacokinetic profile for the three PEG\textsubscript{1000X}-GDCP contrast
agents was similar, differences between the agents may still be detectable in an MRI situation, so all three were tested.

Methods

MR imaging was performed in Sprague-Dawley rats (170-190 g); 3 rats were tested for each agent. Rats were anesthetized by the intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). Contrast agents were injected intravenously at a low dose of 0.03 mmol-Gd/kg via a tail vein. Imaging was performed on a Siemens TrioTim 3T MR scanner. The system body coil was used for rf excitation and a human wrist coil was used for rf reception. A 3D FLASH (FL3D) pulse sequence was used for precontrast image acquisition with the following parameters: $TE = 2.44$ ms, $TR = 7.75$ ms, $25^\circ$ flip angle, two averages, and 0.6 mm coronal slice thickness for 72 slices. After the injection of contrast agent, a high-resolution time of flight MR angiography (TOF-MRA) protocol was implemented with imaging parameters of 25 ms $TR$, 4.23 ms $TE$, one average, and a $25^\circ$ flip angle for 197 axial slices with a saturation band and a resolution of 0.5 mm and 320 row x 512 column imaging matrix. The total acquisition time for the TOF-MRA pulse sequence was just over 30 min. A follow-up FL3D acquisition was applied immediately follow the TOF-MRA pulse sequence to analyze residual contrast enhancement in various organs and tissues.

MR angiographic images (3D) and FL3D images pre- and postcontrast enhancement with each agent were processed using OsiriX software. In the source FL3D images, regions of interest (ROIs) were drawn over the heart, kidney medulla and cortex, liver, and muscle. The contrast to noise ratio (CNR) for the heart, medulla, cortex, and liver was calculated relative to muscle, such that $\frac{SI_{\text{tissue}} - SI_{\text{muscle}}}{SD_{\text{noise}}}$. The standard
deviation of noise was calculated by a 980 voxel area of air in the image. The CNRs for pre- and postcontrast enhancement with the FL3D pulse sequence for each contrast agent were tested for significant differences in each organ by a Student's t-test. A significant difference was considered to be $p < 0.05$.

**Results and Discussion**

Figure 31 shows the resulting 3D-MIP images from the high resolution TOF-MRA pulse sequence after a low dose (0.03 mmol-Gd/kg) of either PEG$_{1000H}$-GDCP, PEG$_{1000M}$-GDCP, PEG$_{1000L}$-GDCP, or GDCP had been injected into a rat tail vein. For each agent, the abdominal aorta can be seen clearly. In addition branching, from the aorta to vessels feeding the liver and also branching in the shoulder region can also be seen. The jugular veins above the heart is enhanced for all agents as this blood flow is descending and therefore selectively enhanced by the TOF-MRA pulse sequence. Contrast agent accumulation can be seen in the renal calyx and renal pelvis for each agent. Drainage by the ureters from the renal pelvis to the bladder is visible for all contrast agents, supporting clearance via renal filtration. Little average signal intensity is present in the liver or muscle for any of the agents because of the TOF pulse sequence. Comparing the 3D-MIP images for each of the agents, all agents produced relatively high enhancement. The small differences observed could be variation between rats or positioning of the rat in the magnet.

FL3D pulse sequences were taken precontrast enhancement and after the TOF-MRA experiment for each contrast agent. Figure 32 shows the CNRs from source images in several organs pre- and postcontrast enhancement from PEG$_{1000H}$-GDCP, PEG$_{1000M}$-GDCP, PEG$_{1000L}$-GDCP, and GDCP. Significant differences were observed in
Figure 31. MRA with GDCP and PEG$_{1000X}$-GDCP. 3D maximum intensity projection (MIP) images of high-resolution TOF-MRA images of PEG$_{1000H}$-GDCP (A), PEG$_{1000M}$-GDCP (B), PEG$_{1000L}$-GDCP (C), and GDCP (D). Each contrast agent was administered intravenously at a dose of 0.03 mmol-Gd/kg.
Figure 32. Precontrast and 30 min postcontrast contrast to noise ratio (CNR) from PEG_{1000H}-GDCP (A), PEG_{1000M}-GDCP (B), PEG_{1000L}-GDCP (C), and GDCP (D). CNRs were calculated by \((S_{\text{tissue}} - S_{\text{muscle}})/S_{\text{noise}}\). Values are given as mean CNR ± SD \((N = 3)\). *\(p < 0.05\) by Student’s t-test.
the heart postcontrast images with PEG\textsubscript{1000H}-GDCP \((p = 0.0011)\). The CNR at 30 min was higher for PEG\textsubscript{1000M}-GDCP and GDCP compared to precontrast CNR, but the differences were not significant. The enhancement as measured by CNR for both the renal cortex and medulla was significant for all for contrast agents, except for the cortex with PEG\textsubscript{1000M}-GDCP. Only for PEG\textsubscript{1000H}-GDCP was the CNR higher in liver postcontrast than in precontrast images.

The TOF-MRA images, selective for arterial flow, are the average of signal produced from the blood pool over 30 min (Figure 31). The descending abdominal aorta can be clearly observed for each of the four contrast agents. For current clinically approved MRI contrast agents, a long pulse sequence like the MRA sequence implemented here would pose a severe limitation, not necessarily because of their relatively short half-life alone, but also because of rapid extravasation out of the blood pool into surrounding tissue. The relatively high concentration of all three PEG-GDCP contrast agents and GDCP over for the first 30 min of the plasma pharmacokinetics (Chapter 6), translates into a low distribution volume (Table 3), and results in excellent contrast enhancement in a relatively long MRA pulse sequence. Small vessels that branch into the liver, shoulder, and neck regions are also clearly visible. This further suggests that the distribution of these agents is limited to mainly the blood pool and the agents do not readily diffuse into tissue over the course of the experiment. Enhancement in the ureters supports that these agents are getting cleared via renal filtration.

**Summary**

The potential of PEG-GDCP for angiographic MR imaging was evaluated in this chapter. At a low dose (0.03 mmol-Gd/kg) each of three PEG-1000 grafting ratios and a
similar sized GDCP contrast agent showed excellent enhancement of vasculature in a TOF-MRA protocol. The significant amount of signal present at 30 min in the FL3D pulse sequence suggests that plasma retention of these agents is sufficient for longer MRA exams. Both PEG\textsubscript{1000}-GDCP and GDCP showed impressive imaging properties in this study. However, PEG\textsubscript{1000}-GDCP had minimal long-term Gd(III) accumulation (Chapter 6), which suggests that it would be an ideal contrast agent for further development as will be discussed in Chapter 8.

**Literature Cited**


Magnetic resonance imaging (MRI) is a powerful diagnostic imaging modality that can detect and analyze potentially pathologic morphology and physiology. Approximately 30% of MR examinations are contrast enhanced by low molecular Gd(III) complexes to further delineate and characterize diseased tissue. These complexes are stable, but have suboptimal pharmacokinetic performance, which limits the number of potential applications, especially where enhancement of the vascular network is desired. The development of macromolecular Gd(III) complexes is a clear alternative because macromolecules have a distribution more limited to the intravascular space with extended circulation times. Their efficacy over low molecular weight Gd(III) contrast agents has been well demonstrated in preclinical studies. Unfortunately the clinical development of these agents is severely limited due to their potential toxicity from long-term Gd(III) accumulation.

Safety is the chief priority in the development of any probe for diagnostic imaging. An ideal contrast agent would have the blood pool imaging properties of a macromolecular contrast agent, but after the length of the exam be readily cleared with minimal long-term tissue accumulation. Preliminary results with GdDTPA cystamine copolymers (GDCC) showed that these macromolecular contrast agents could be readily
degraded via the thiol-disulfide exchange reaction in the presence of a free thiol. In vivo, these contrast agents provided more persistent enhancement than a control agent due to their macromolecular properties, but were then gradually degraded and cleared via renal filtration resulting in minimal long-term Gd(III) accumulation. More prolonged contrast enhancement is required, however, for longer imaging protocols, such as MR angiography. Structural modification around the disulfide bond by PEGylation was hypothesized to increase distribution to the intravascular space for improved contrast enhancement, but still provide minimal long-term Gd(III) accumulation. Several specific aims were undertaken to assess this hypothesis: (1) Synthesize and physiochemically characterize PEGylated biodegradable Gd(III) polymeric contrast agents for MR imaging; (2) Determine relaxation properties and (3) in vitro degradation of the contrast agents; (4) Perform preliminary contrast enhanced MRI with the contrast agents in mice; (5) With a lead agent from the preliminary studies, investigate the pharmacokinetic and long-term Gd (III) properties of the agent; and (6) Investigate the potential for these contrast agents for application in MR angiography.

PEG-g-poly(GdDTPA-co-L-cystine) (PEG-GDCP) was synthesized by conjugating MPEG-NH₂ to the carboxylate groups of poly(GdDTPA-co-L-cystine) (GDCP) via NHS and EDC. PEG-GDCP was synthesized with several variations of molecular weight and PEG grafting degrees. The physiochemical parameters are summarized in Table 2 (page 71). Overall, the relaxivity of the contrast agents seemed rather invariable to the physiochemical characteristics of the contrast agent, including molecular weight and PEG grafting characteristics. Grafting of PEG-2000 had a larger effect on relaxivity compared to PEG-1000, although the difference is not expected to be clinically significant.
The first series of contrast agents had PEG-2000 grafted in a high and low ratio to GDCP, PEG\textsubscript{2000H}-GDCP and PEG\textsubscript{2000L}-GDCP, respectively. Preliminary testing in healthy mice at a dose of 0.03 mmol-Gd/kg revealed that the two PEGylated agents gave superior contrast enhancement in both duration and signal intensity compared to a clinical control Gd(DTPA-BMA). The contrast enhancement from PEG\textsubscript{2000H}-GDCP appeared to be greater than PEG\textsubscript{2000L}-GDCP, but the contrast enhancement from both had dissipated after 60 min.

To characterize the effect of PEGylation on the biodegradability of the agents via the thiol-disulfide exchange reaction, the PEGylated macromolecular contrast agents were incubated with a free thiol, L-cysteine. PEG\textsubscript{2000H}-GDCP (PEG-2000 grafting, high grafting degree) showed slightly less susceptibility to degradation a thiol than PEG\textsubscript{2000L}-GDCP (PEG-2000, low grafting degree) after 3 h, but after 24 h, PEG\textsubscript{2000L}-GDCP was completely degraded, whereas PEG\textsubscript{2000H}-GDCP showed little additional degradation, suggesting that the degree of PEG grafting may play a role in the controlled degradation of these agents.

It was deduced from the first in vivo series of experiments that PEG likely contributed to increased vascular retention of these agents, possibly by increasing the size of the agents or decreased accessibility to the disulfide bond. Based on the results of the enhancement characteristics and slower in vitro degradation, PEG-GDCP of higher grafting degree appeared to be more favorable for imaging of the blood pool. A high PEG grafting ratio was subsequently selected for development of the next series of PEG-GDCP contrast agents.
To further characterize the effect of PEG grafting for optimal contrast enhanced MR imaging – a balance between maximal signal intensity for the length of the exam and minimal long-term Gd(III) accumulation – the chain length of the PEG graft was varied. The length grafted PEG was either 2000 Da, 1000 Da, or 550 Da. In vitro, degradation with increasing concentrations of L-cysteine revealed that PEG\textsubscript{2000}-GDCP was the least susceptible to degradation by a thiol, while PEG\textsubscript{550}-GDCP was most susceptible to degradation and PEG\textsubscript{1000}-GDCP had a degradation profile in between. In vivo, the agents were tested in healthy mice. The results in mice match closely with those predicted in vitro with the degradation experiments of the agents. PEG\textsubscript{2000}-GDCP provided the most prominent enhancement in the blood pool. PEG\textsubscript{1000}-GDCP and PEG\textsubscript{550}-GDCP were cleared away more quickly, but the enhancement of PEG\textsubscript{1000}-GDCP was more sustained.

Despite the better image quality of PEG\textsubscript{2000}-GDCP from the second series of experiments, it was not chosen as the lead agent for further development in animal tissue experiments and as an agent for MR angiography. As stressed throughout this dissertation, an ideal contrast agent must have the proper balance between excellent blood pool enhancement characteristics and safety, one marker of which is minimal long-term Gd(III) accumulation. In the second series of preliminary studies, it was suspected that the degradation products of PEG-2000 grafted GDCP may have some limitation in its clearance as demonstrated by the signal intensity curve in the kidney. As a result PEG\textsubscript{1000}-GDCP was selected for further studies because it still provided high-quality contrast enhancement, sufficient for a longer imaging protocol, but its signal intensity rapidly approached baseline levels after one hour.
The pharmacokinetic and long-term Gd(III) retention profiles of PEG\textsubscript{1000}-GDCP were tested in Sprague-Dawley rats. Three ratios of PEG-1000 grafting were synthesized to better understand the effects of PEGylation in vivo and compared against GDCP of similar molecular weight. In the first 15 min postinjection of contrast agents, each PEG-GDCP contrast agent had a higher plasma concentration than GDCP. After 30 min, however, the three PEGylated contrast agents were readily removed from the plasma. These results were consistent with other polydisulfide contrast agents where a neutral or amide bond was present surrounding the disulfide by modification of the carboxylate group. One potential explanation is that by converting negatively charged GDCP into a neutral polymer, this may reduce electrostatic repulsion between the contrast agent and of negatively charged thiolate anion conjugate bases from glutathione, cysteine, etc. in the plasma.

Considering the deposition of Gd(III) ions after the intravenous injection of the three PEG\textsubscript{1000}-GDCP contrast agents and GDCP, the rapid elimination of PEG-GDCP contrast agents resulted in minimal tissue accumulation with little difference between the three agents. GDCP consistently showed higher accumulation in tissues after ten days than the PEG-GDCP contrast agents. This difference was most notable in the liver, where the percent remaining of Gd(III) remaining of rats injected GDCP was 10 times higher than the PEG-GDCP contrast agents. Charge likely has a governing role for the difference in accumulation in the liver. Polyanions that are exogenous to the body can be readily picked up by scavenger cells in the liver and subsequently metabolized. PEGylation of GDCP, which masks charge, combined with more rapid clearance of PEG-GDCP most likely accounts for lower PEG-GDCP accumulation in the liver.
Urine was also collected after the administration of PEG_{1000}-GDCP and GDCP and was analyzed by MALDI-TOF MS after 8 h of collection. Gd(III) isotope patterns were observed in both positive- and negative-charge-labeled mass spectra. Identification of the exact structure corresponding to a peak was difficult due the likelihood of further metabolism of the degradation unit, other degradation mechanisms than just the thiol-disulfide exchange reaction, and the potential presence of multiply charged oligomers. The metabolite peaks indicate that PEG was eliminated from the GDCP backbone. This was not observed in the in vitro degradation profile with a free thiol, which suggests that in vivo degradation of the polydisulfides is more complex than only reduction of the disulfide. What can be concluded from the metabolic analysis is that a majority of the injected Gd(III) dose was collected in the first 8 h and that Gd(III) was cleared as small intact complexes. From the pharmacokinetic experiments, PEGylation of GDCP resulted in a polymer with an increased distribution to the plasma for the first 15 min after bolus intravenous injection. After a half hour, a more than sufficient duration for most contrast enhanced MRI exams, the rapid clearance of PEG_{1000}-GDCP resulted in the desired minimal accumulation of Gd(III) ions.

The potential of PEG_{1000X}-GDCP for angiographic pulse sequences was verified in rats. The TOF-MRA pulse sequence for this experiment was approximately 30 min. The polymeric contrast agents were injected at dose of 0.03 mmol-Gd/kg, less than one-third of the standard clinical dose. All agents showed excellent enhancement of the aorta and common iliac arteries. Branching arteries from the aorta, vessels of liver and those of the shoulder and neck region could also be seen. The excellent contrast enhancement from
PEG_{1000}-GDCP combined with minimal long-term Gd(III) accumulation make it an ideal contrast agents for further development.

**Future Direction**

The results from this body of work demonstrate the potential utility of PEG-GDCP for further development. These contrast agents have shown excellent contrast enhancement in dynamic and high-resolution angiographic pulse sequences. A lead agent PEG_{1000}-GDCP showed initially higher plasma concentrations and a smaller distribution volume than GDCP, but minimal long-term Gd(III) accumulation. These properties are closely related to what is most sought after for a blood pool MRI contrast agent. There are several potential directions for the future development of PEGylated biodegradable macromolecular contrast agents. At this point, two clear directions for further investigation are possible based in the results presented in this work. First, a more in depth understanding of the exact mechanism of polydisulfide degradation is needed, including the role of structural modification around the disulfide bond and deeper understanding of the physiochemical nature of these Gd(III) complexes. Second, since the in vivo MRI results are very promising, a prudent next step would then be to apply PEG-GDPCP contrast agents to disease models of cancer and the cardiovascular system.

**In Depth Structural Characterization of PEG-GDCP**

A more in depth study for the mechanism of relaxivity of the contrast agents is needed. It was hypothesized in Chapter 3 that PEG conjugation may interact with Gd(III) water exchange because of the large hydrogen bonding capacity of PEG. In addition, the grafting degree of PEG-2000 had a larger effect on relaxivity than agents modified with PEG-1000. To better understand this phenomenon, more detailed experiments are
Elucidation of Thiol-Disulfide Exchange Mechanism In Vivo

MALDI-TOF MS revealed that the degradation of PEG-GDCP in vivo is much more complicated than what was observed in vitro. In vitro, there was mostly reduction via the thiol-disulfide exchange reaction into the expected degradation products. The in vivo metabolites detected in urine suggested the PEG was removed from the backbone. In addition, metabolites that had characteristic Gd(III) patterns, were different from those expected. We hypothesized that more complicated degradation, including reduction and oxidation and further metabolization of the degradation products, occurs in vivo. Because of the difficulty of isolating the metabolites from urine, it may be possible to incubate the polydisulfide complexes with suspected reductases or oxygenases such as CDO. The enzyme could be separated from degradation product by size exclusion chromatography or ultrafiltration. This would potentially yield isolated degradation products that could be characterized by more comprehensive analytical techniques, such as LC/MS or MS/MS. Accurate characterization of the degradation products may give insight into the mechanism of degradation.

needed. A detailed relaxometric analysis on the polydisulfide systems would include the following: recording and analysis of NMRD profiles from 0.01 to 120 MHz, measurement of $T_2$ from 20 to 80 MHz, and $^{17}$O VT/T$_2$ for exchange lifetimes of the coordinated water. In addition, light scattering studies may provide more information on the exact molecular weight of the compounds instead of just apparent molecular weight, which was determined in the experiments presented here.
Application of PEG-GDCP to Animal Disease Models

Tumor Imaging

Cancer patient survival is inversely related to tumor angiogenesis and vascularity (1). As a result, the ability to detect and characterize tumors is necessary for optimal treatment to increase the chance of patient survival. Contrast enhanced MRI is repeatable because it is minimally invasive, which allows the potential to detect and stage tumor tissue, measure angiogenesis, and to assess the therapeutic efficacy of cancer treatment (2,3). In preclinical studies, macromolecular Gd(III) complexes were more effective at characterizing animal tumors than low molecular weight Gd(III) chelates (4-6). As discussed in Chapter 1, the development of macromolecular Gd(III) complexes is limited by the toxicity associated with long-term Gd(III) accumulation. It was determined in the experiments presented here that PEG-GDCP shows more prominent enhancement of the blood pool than a low molecular weight agent, but has more favorable Gd(III) clearance compared to the results of currently developed macromolecular MRI contrast agents. Therefore, PEG-GDCP may be an ideal agent for characterizing tumor tissue because its macromolecular characteristics could accurately assess the tumor, but the agent would not have the limitations of long-term Gd(III) accumulation.

Cardiovascular Disease Imaging

Cardiovascular disease is a leading cause of death worldwide. Vulnerable atherosclerotic plaque is the predecessor to more severe complications such as carotid artery disease (7,8). The degree of arterial stenosis is not enough to fully characterized vascular plaque, but the plaque composition may be a more critical indicator of cardiovascular disease (9). Biodegradable macromolecular Gd(III) complexes would
have definite utility in the characterization of plaque vulnerability because like cancer, microvessels are also involved in the development of plaque lesions, where microvessel formation indicates a likelihood of plaque rupture. Thus, PEG-GDCP would provide a safer alternative than currently developed macromolecular Gd(III) complexes and potentially a more accurate diagnosis than clinically approved low molecular weight Gd(III) complexes.

Literature Cited


