Monitoring of Immune Cell Response to B Cell Depletion Therapy and Nerve Root Injury Using Spio Enhanced MRI

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Monitoring of Immune Cell Response to B Cell Depletion Therapy and Nerve Root Injury Using Spio Enhanced MRI

Abstract
Magnetic resonance (MR) is a robust platform for non-invasive, high-resolution anatomical imaging. However, MR imaging lacks the requisite sensitivity and contrast for imaging at the cellular level. This represents a clinical impediment to greater diagnostic accuracy. Recent advances have allowed for the in vivo visualization of populations and even of individual cells using superparamagnetic iron oxide (SPIO) MR contrast agents. These nanoparticles, commonly manifested as a core of a single iron oxide crystal or cluster of crystals coated in a biocompatible shell, function to shorten proton relaxation times. In MR imaging these constructs locally dephase protons, resulting in a decrease in signal (hypointensity) localized to the region of accumulation of SPIO. In the context of immune cell imaging, SPIO can provide insight into the cellular migration patterns, trafficking, temporal dynamics and progression of diseases and their related pathological states. Furthermore, by visualizing the presence and activity of immune cells, SPIO-enabled cellular imaging can help evaluate the efficacy of therapy in immune disorders.

This thesis examines the production, modification and application of SPIO in a range of in vitro and in vivo immune-response-relevant cellular systems. The role of different nanoparticle characteristics including diameter, surface charge and concentration are investigated in the labeling of T cells in culture. Following optimization of SPIO loading conditions for lymphocytes, the effect these particles have on the activation of primary B cells are elucidated. B cells are tracked using a variety of modalities, with and without the application of B cell depleting therapy. This is to evaluate the efficacy of SPIO as in vivo marker for B cell distribution.

Unmodified SPIO were applied to monitor macrophage infiltration in a transient nerve root compression model, with implications for neck pain diagnosis and treatment. Nanoparticle accumulation and MR hypointensity was correlated to the presence of activated macrophage at the site of injury. Taken together, the application of SPIO to study nanoparticle uptake in vitro and visualization of immune cells in vivo provide a basis for advanced study and diagnosis of diverse pathologies.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Bioengineering

First Advisor
Dr. Andrew Tsourkas

Keywords
Nanoparticles, Cellular and Molecular Imaging, Cell tracking

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Daniel L.J. Thorek

A DISSERTATION

In

Bioengineering

Presented to the Faculties of the University of Pennsylvania in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2009

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MONITORING OF IMMUNE CELL RESPONSE TO B CELL DEPLETION THERAPY
AND NERVE ROOT INJURY USING SPIO ENHANCED MRI

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2009

Daniel L.J. Thorek
DEDICATION

This thesis is dedicated to Carolyn S. Jaffe. Without her, this work would have literally been impossible. It would have also been much less enjoyable. I thank her for the sage advice, the inexhaustible kindness and the sanctuary.
ACKNOWLEDGEMENTS

I wish to thank my thesis advisor, Dr. Andrew Tsourkas, for the opportunity to work in his laboratory. My first several months at the University of Pennsylvania were unexpectedly difficult. Fortuitously, I was provided the chance to be exposed to a fascinating new field and the mentorship of an enthusiastic and dedicated researcher. Over the past 5 years Andrew has provided me with the freedom, responsibility and resources to try, create, fail, learn and succeed.

I would also like to thank the members of my thesis committee; Drs. Eisenberg, Winkelman and Zhou. The first two members afforded me the ability to conduct collaborations in their fields of expertise and Dr. Zhou was helpful in discussing MR related issues. The early home and continuing advisory role that I found in lab and person of Dr. Crocker was deeply appreciated. I am also indebted to the technical staff of the Small Animal Imaging Facility; Drs. Pickup, Weixia Liu and Yvette Liu. They were instrumental in instrumentation and were always willing to answer my 'one more question'.

My peers have been a source of constant assistance, one that I can only hope has been reciprocated. Doctoral candidates in the Cellular and Molecular Imaging Lab of the same cohort, Julie Czupryna and Dr. Chen, have made my research and personal
experiences more fruitful than they otherwise would have been. Other current and former lab members to which I must extend my thanks include Dr. Zheng, Lanlan Zhou, Dr. Lee, Drew Elias, Sam Crayton, Rob Warden and the many undergraduate students that worked in the lab on nanoparticle related projects. Members of affiliated laboratories that helped me in my adventure include Christine Weisshaar, Drs. Jao, Arora, Rothman and Tsao.

The encouragement of my friends and family was deeply felt throughout my career as a graduate student. The sustained support of my siblings and parents was always welcome and sometimes necessary. Finally, I would like to acknowledge the unwavering patience, virtue, support, love and patience of my partner April Kelly. I would certainly not have accomplished this degree without her. Another Borges quote; *Being with you and not being with you is the only way I have to measure time.*
ABSTRACT

MONITORING OF IMMUNE CELL RESPONSE TO B CELL DEPLETION THERAPY AND NERVE ROOT INJURY USING SPIO ENHANCED MRI

Daniel L.J. Thorek

Andrew Tsourkas, Thesis Advisor

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2. **Equation 1.2**
   \[ v = \gamma \cdot B \]

3. **Equation 1.3**
   \[ E = h\nu \]

4. **Equation 1.4**
   \[ E = h \cdot \gamma \cdot B \]

5. **Equation 1.5**
   \[ M_z = M_0 \left(1 - e^{-\gamma T_1}\right) \]

6. **Equation 1.6**
   \[ M_{xy} = M_{xy0} \left(e^{-\gamma T_2}\right) \]

7. **Equation 1.7**
   \[ CNR = \frac{(SI_1 - SI_2)}{\text{noise}} \]
EQUATION 1.8
\[ R_i = \Delta C/\Delta T_i \]

EQUATION 1.9
\[ E_a = K_a V \]

EQUATION 1.10
\[ \tau_N = \tau_0(E_a) e^{E_a/kT} \]

EQUATION 1.11
\[ \tau_B = \frac{3V\eta}{kT} \]

EQUATION 1.12
\[ \frac{1}{\tau} = \frac{1}{\tau_N} + \frac{1}{\tau_B} \]

EQUATION 1.13
\[ Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O \]

EQUATION 1.14
\[ Fe_3O_4 + 2H^+ \rightarrow \gamma Fe_2O_3 + Fe^{2+} + H_2O \]

EQUATION A.1
\[ f_{R_i} = \frac{q \cdot \mu_{eff}^2 T_c}{r^6} \]

EQUATION A.2
\[ \frac{1}{T_c} = \frac{1}{T_r} + \frac{1}{T_{r_e}} + \frac{1}{T_m} \]
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CHAPTER 1. Magnetic Resonance Imaging, SPIO, and Cell Tracking

1.1 - Overview

Magnetic resonance (MR) imaging is a powerful diagnostic platform in widespread clinical use. MR demonstrates visualization of healthy and disease relevant features in both time and space through the display of high-resolution anatomical information. Image formation is dependent on the intrinsic contrast of adjacent tissues. This distinction in signal is provided by the different rates of realignment of hydrogen nuclei within an external magnetic field, following a radio frequency pulse sequence. However, the modality is often restricted as a result of low sensitivity to changes between tissues and tissue states.

The use of MR for molecular and cellular imaging (MCI) is an emerging subfield with significant potential to impact clinical and research applications. MCI has been defined as the non-invasive, quantitative and repetitive imaging of biomolecules and biological processes in living organisms (1). Unfortunately, the applicability and translation of MCI approaches, such as more accurate, earlier detection of disease or the study of biological phenomena, is hindered by MR imaging's inherently low signal-to-noise ratio. This deficiency is mitigated through the use of contrast agents (CA). Unlike direct signal detection or interference as caused by CA in x-ray imaging applications, MR
CA function by altering characteristics of surrounding water protons through their magnetic properties.

This chapter will provide a brief overview on the fundamentals of MR and recent advancements in the disciplines relevant to cellular magnetic resonance imaging. First, an overview of the quantum mechanical principles underlying nuclear spin relaxation will be discussed. Image generation for biological samples will be described. This will be followed by an examination of issues of tissue contrast and a description of T2* contrast agents. A detailed overview of the physical and biological properties of superparamagnetic iron oxide nanoparticle agents will be given. Finally, the imaging approach used in the tracking and identifying of immune cells for biomedical purposes will be discussed. A special emphasis will be placed on the application of SPIO particulates for immune and inflammatory cell relevant imaging.
1.2 - Nuclear Magnetic Resonance and Spin Relaxation Processes

Magnetic resonance (MR) is a versatile instrument for non-invasive imaging of living specimens in the clinic and in research. In the United States alone, there were over 26 million MR procedures at approximately 7000 hospital and non-hospital MR imaging sites in 2007 (2). This imaging modality is based on the same principles as its forbearer in research, nuclear magnetic resonance spectroscopy (NMR). As the vast majority of clinical research on MR and all of the work detailed in this thesis relate exclusively to the hydrogen nucleus, the description of MR imaging relates only to proton ($^1$H) MR. However, the fundamentals discussed below can be extended to MR studies of other nuclei with quantum spin numbers of 1/2.

All quantum particles have the property of spin, a fundamental property of nature like electrical charge or mass. The proton, with its unmatched positive charge, can be thought of a spinning charged particle with a spin of 1/2. Spinning charged particles, similar to rotating electrically charged bodies, have a magnetic dipole (north and south) and MR imaging is predicated on the manipulation of this dipole through the use of radio waves in a static magnetic field.

The nuclear spin angular momentum, or spin, of $^1$H can be described as ($S$), a vector that can be quantified in Equation 1.1. Here, $h$ is Planck’s constant and $s$ is the nuclear spin quantum number. For the proton $s$ is equivalent to 1/2.

$$1S = \frac{h}{2\pi} \sqrt{s(s+1)}$$

Equation 1.1

When placed in an external magnetic field of strength ($B_0$) the spin vector ($S$) of the proton aligns in the direction of the applied field. This 'atomic magnet' is now said to be
in a low energy state, as it is aligned with the applied field. The proton can undergo a transition to a high-energy state (anti-parallel to the applied field) through the absorption of a photon of specific energy. In quantum mechanics the energy transferred has a discrete value, ie. it is quantized.

The energy of this photon is equivalent to the energy difference between the two states; parallel (or aligned) and anti-parallel. Protons can absorb photons that have a frequency, $\nu$, as defined in Equation 1.2. The gyromagnetic ratio of the particle, $\gamma$, for $^1$H is equal to 42.58 MHz/T.

$$\nu = \gamma \cdot B$$

Equation 1.2

This frequency, $\nu$, is known as the Larmor or resonant frequency of the nuclei. The frequency of the photon can be converted to energy through the wave-particle duality as described in Equation 1.3.

$$E = h \nu$$

Equation 1.3

This allows for the calculation of the energy necessary (through absorption) to switch a proton from the high to low energy state. This is shown in Equation 1.4.

$$E = h \gamma \cdot B$$

Equation 1.4

The field strength of an MR must be high in order to align the weak magnetic dipole moment of $^1$H. As field strength increases, the spectral resolution and sensitivity increase, resulting in improved signal to noise as the energy difference between states increases. At high field, photon frequencies for excitation to alter nuclear spin states are in the radiofrequency (RF) range (MHz).

Moving from a single proton or a single magnetic dipole moment to a collection of spins, first randomly aligned as seen in Figure 1.1A and then aligned with an external
field $B_0$ along the $z$-axis, we see the emergence of a net magnetization vector ($M_z$) in Figure 1.1B.
(A) The spinning and precessing nuclei generate a magnetic dipole moment (red). These moments are randomly aligned in the absence of an external magnetic field. (B) With the application of that field ($B_0$) the net magnetization the dipole moments generates an equilibrium net magnetization vector ($M_0$). At equilibrium, this is equal to the z-axis component of the magnetization vector ($M_z$, blue).
With an applied magnetic field along the z-axis a net magnetization vector develops, aligned with the applied field. This net vector ($M_z$) increases when placed in the field as the $^1$H become preferentially aligned in the low energy state until reaching an equilibrium distribution between the states. It is then equal to the equilibrium magnetization vector ($M_0$). Further, there is no net magnetization of protons' magnetic dipoles in either the x- or y-directions. With the application of a RF pulse equal to the Larmor frequency of the precessing, aligned nuclei, the z-component of the net magnetization vector can be reduced to zero. As the system relaxes back to a lower energy state (globally) after the removal of the radiofrequency source, $M_z = 0$ returns back to the equilibrium condition $M_z = M_0$. The recovery of the longitudinal magnetization is described in Figure 1.2.
Figure 1.2 - Longitudinal Magnetization and T₁.

(A) Following the application of a radiofrequency pulse at the Larmor frequency, the net magnetization is saturated and no longer possesses a longitudinal bias. Immediately following the removal of the radio energy, the system returns to its lower energy state, with a majority of ¹H aligned with the field. (B) The growth of the z-component magnetization vector (Mz) back to a fraction (1-1/e or 63%) of the equilibrium state is described by the time constant T₁. The time (t) refers to time after removal of radiofrequency.
Equation 1.5 describes the $T_1$ or spin lattice relaxation time, a value used to describe the exponential recovery of $M_z$. $T_1$ is a constant that is equal to the time required for the recovery of 63% of the longitudinal magnetization.

$$M_z = M_0\left(1 - e^{-\frac{t}{T_1}}\right) \quad \text{Equation 1.5}$$

The net magnetic moment can be placed, or flipped, into the orthogonal xy-plane through the application of specific radiofrequency pulses that result in phase coherence of the magnetic dipoles of the $^1$H nuclei. This means that an xy-component of the net moment ($M_{xy}$) is generated and this will rotate about the axis of the applied field. The frequency of this rotation will be equal to the Larmor frequency (3).

This state does not last following the removal of the pulse, as perturbations in the local magnetic field by other nuclei as well as imperfections in the applied field lead to reduction and finally absence of coherence. As the transverse magnetization vector rotates about the applied field, the $M_{xy}$ will decrease as illustrated in Figure 1.3. The decrease in transverse magnetization can be described by $T_2$ or the spin-spin relaxation time. This is the time needed for $M_{xy}$ to be reduced by 63%. Mathematically, this is expressed in Equation 1.6. It must be stressed that the $T_1$ and $T_2$ relaxation are independent processes. They are related through the bulk direction of net magnetic dipole moment and occur simultaneously but describe separate physical events.
Figure 1.3 - Transverse Magnetization and $T_2$.

The net magnetization vector can be flipped into the transverse plane whereupon it is denoted $M_{xy}$, green. In (A), following the removal of the radiofrequency pulse the vector continues to rotate around the direction of the applied field, indicated by the arrow head on the perimeter path of the transverse vector. The rapid loss of phase coherence leads to the decay of $M_{xy}$. In (B), the time constant of decay of magnetization, spin-spin relaxation or $T_2$ is shown. Please note the change in frame of reference.
In an ideal system, all dephasing of spins would begin with all nuclei precessing at the same phase. However, in application the field is never fully homogeneous, and in all biological samples of interest there are chemical differences that disturb the phases of $^1$H. This leads to enhanced non-random relaxation effects that are termed $T_2^*$ decay. The inverse of $T_2^*$ is equal to the summation of the inverse of the $T_2$ decay and the dephasing produced by inhomogeneities.

To summarize, magnetic resonance is based on the electromagnetic properties of hydrogen nuclei. Their nuclear spin, coupled with their charge, creates a magnetic dipole moment. In bulk, the aggregate magnetic moment or net magnetization is zero in the absence of a magnetic field. With the application of a field, a majority of protons align with the field, generating a net magnetization vector aligned with the applied field. This net magnetization can be manipulated by switching protons from a low energy (aligned) to a higher energy (anti-parallel) state with the application of electromagnetic radiation of a specific energy. For the purposes of MR imaging this is in the RF range. Following the removal of the radio source, the net magnetization returns to equilibrium through two forms of relaxation, $T_1$ and $T_2$. The former sees nuclei return energy to the surrounding lattice or medium as the nuclei re-align preferentially with the applied field. In the latter process, transverse magnetization decreases as nuclei lose phase coherence (as spins must be in phase in the transverse direction to be detected) and through nuclei exchanging energy with their neighbors.

$$M_{xy} = M_{xy0} \left( e^{-\frac{t}{T_2^*}} \right)$$  \hspace{1cm} \text{Equation 1.6}
1.3 - Magnetic Resonance Image Formation

For clinical and research applications, the samples of interest are heterogeneous and large. MR information that is relevant for diagnostic purposes requires that spatial information be encoded into the signal in three directions; x, y and z. The discussion here will focus on the generation of 2-dimensional MR images, as acquired for analysis in this dissertation.

Spatial encoding is accomplished by time-varying the strength of the magnetic fields. In the simplest case, a one-dimensional linear gradient (a variation of the magnetic field with respect to position) along the direction of the applied field is created, termed the Gz or z-gradient, as shown in Figure 1.4. Now each hydrogen nuclei at a distance \( z_i \) along the gradient precesses at a unique frequency. This frequency can be determined for each local field strength along the gradient, as previously shown in Equation 1.2. This will be termed the slice selection gradient, as we are interested in imaging a plane perpendicular to the applied gradient.
Figure 1.4 - Slice Selection.

(A) A magnetic gradient (dashed line) is applied along the z-direction. This results in each plane perpendicular to the z-axis experiencing a different magnetic field. Thus, at each $z_i$, protons precess at different angular frequencies. These frequencies, $\omega_i$, vary proportionately with their distance along the gradient, as the applied gradient is linear.
(B) This can be exploited for slice selection purposes by exciting a plane selectively with a radiofrequency pulse of a power and bandwidth required to flip the $^1$H spins in the desired plane. The number of arrows in the cartoon body, which differ along the gradient, symbolize the field strength experienced at different anatomical locations.
The next step in the imaging process is to spatially encode within the selected slice using gradients applied perpendicular to the slice selection gradient. There are two remaining directions, the x and the y, in which MR signal needs to be localized. First, phase encoding is performed. This is accomplished with the brief application of a gradient, Gy, in order to align the phases of the spinning nuclei within the slice, according to their position relative to the gradient. This brief dephasing results in a slice whose nuclei all spin at the same frequency, but at different phases relative to their location within the slice. In conventional, supine, MR this gradient is applied anterioposterially. As shown in Figure 1.5A this results in phase encoded nuclei that spin in a phase proportional to their position in the gradient in the transverse plane. In other words, the Larmor frequency for proton spin along the gradient is different at each position along that gradient.

The third and final spatial encoding element is obtained through application of a frequency encoding gradient. This results in $^1$H that precess at a rate that is equal for all nuclei at a given distance along the applied frequency encoding gradient direction, as shown in Figure 1.5B. This gradient is applied laterally, or in Gx for the employed coordinate system, and is switched on during the acquisition of data. For this reason the Gx gradient is also referred to as the readout gradient. The gradient is on during the acquisition, resulting in a spectrum of frequencies (at each distance along the x direction) being read, rather than a single frequency.

This signal is received by radiofrequency coils that surround the subject. The magnetization direction and magnitude of spins at locations within a subject are relayed
by these antennae as these magnetic fields will generate current. In order to determine the x-position, the radiofrequency spectrum must first be analyzed using a Fourier transform. This operation decomposes the spectrum into component frequencies for each position along the applied gradient. The y-position is determined by analyzing the phase distribution within each frequency. This requires that a second transform be employed as the acquired data is the sum of all spins with the same frequency but different phases (schematically within the double-lines in Figure 1.5B). The phases and thus the y-location of the spins can be determined after all rows in a given slice are analyzed.
Figure 1.5 - Phase Encoding.

(A) A phase encoding gradient, Gy, is applied and aligns the frequency of spins perpendicular to the axis of the gradient (i.e. in rows at equal distances along the gradient). The gradient is symbolized to be increasing with opacity of the Gy arrow. (B) The frequency encoding gradient, Gx, is applied during the readout of the resonance information. This gradient encodes the frequency, or rate of precession, in the selected slice plane for all protons experiencing the same frequency encoding magnetic field strength (for example within the double-lines). The gradient is symbolized to be increasing with opacity of the Gx arrow. For clarity - decreased, nominal and increased frequency of precession is noted at the top.
1.4 - MR Imaging Contrast

MR as a modality for diagnostic imaging is dependent on its ability to resolve distinct tissues and physiological features deep within opaque organisms. The ability to discern between tissues and tissue states for imaging purposes is defined by the contrast to noise ratio (CNR; Equation 1.7).

\[ CNR = \frac{SI_1 - SI_2}{\text{noise}} \]  

Equation 1.7

Here, SI is the signal intensity of adjacent regions in an image and these values rely on the earlier discussion of relaxation properties. To recapitulate - \( T_1 \) is a description of recovery of longitudinal magnetization (recovery of 63% of the equilibrium net magnetization) while \( T_2 \) is a measure of the decay of the transverse magnetization (loss of 63% of \( M_{xy0} \)).

In non-ideal systems, additional effects enhance the rate of decay of transverse magnetization, termed \( T_2^* \) decay. Following removal of the RF energy, the relaxation and decay processes initiate immediately. In biological imaging systems there always exist some magnetic inhomogeneities, a result of both the field consistency and its interaction with the sample. These magnetic inhomogeneities serve to enhance the rate of decay of transverse magnetization. \( T_2^* \) decay is thus the combination of \( T_2 \) decay added to the dephasing due to these inhomogeneities. The time for \( T_1 \) is always longer than \( T_2 \), which is always longer than \( T_2^* \).

The differences in \( T_1 \), \( T_2 \) and proton density within a sample determine the intensity of signal (SI) in a given region of a 2D image and therefore its discernment. Using different radiofrequency (RF) and magnetic gradient pulse algorithms, hereafter
called sequences, the tissues' contrast can be enhanced towards one of the three inherent contrast parameters. This is possible because the $T_1$, $T_2$ and proton densities of tissues (and noise) differ, as summarized in the relaxation time values in Table 1.1 and can be exploited for the purposes of contrast throughout the body.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$T_1$ (msec)</th>
<th>$T_2$ (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>576 ± 30</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>1008 ± 20</td>
<td>44 ± 6</td>
</tr>
<tr>
<td>Heart</td>
<td>1030 ± 34</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>Kidney</td>
<td>690 ± 30</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>Blood</td>
<td>1441 ± 120</td>
<td>327 ± 4</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>745 ± 37</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>White matter</td>
<td>884 ± 50</td>
<td>72 ± 4</td>
</tr>
<tr>
<td>Grey matter</td>
<td>1124 ± 50</td>
<td>95 ± 8</td>
</tr>
</tbody>
</table>

Table 1.1 - $T_1$ and $T_2$ Values for Human Tissues at 1.5 T

Accepted values for the $T_1$ and $T_2$ of commonly MR investigated tissues. This table was adapted by Thorek, from (4).

It is useful to examine the resonance response of a single tissue. The longitudinal magnetization recovery of a tissue after a 90° pulse (or flip into the transverse plane) is shown in Figure 1.6A. Here we see the saturation of the signal with the 90° flip to zero and then its exponential recovery dependent on its $T_1$ value. In (B) we see that different tissues or different tissue regions have variable $T_1$ values and thus can be distinguished by selecting appropriate TR, repetition time, and TE, echo time, to sample the signal. The TR is a time value that describes the time from the application of one RF pulse to the
application of the next RF pulse. It is measured in milliseconds (msec). The TE is the
time from the application of the RF pulse, to the peak of the signal induced in a receiver
coil and is also measured in msec. Nearly all imaging protocols sample the signal at this
echo time (peak signal). The TE determines how much decay of transverse magnetization
is allowed to occur before the signal is read.

Visualization of tissue contrast based on $T_2$, or $T_2$-weighting ($T_2$-w), can be
accomplished by exploiting the equivalent $T_2$ differences between tissues as shown for
$T_1$-w imaging above. This imaging mode is described in Figure 1.7. Care must be taken
to note the superimposition of the $M_{xy}$ decay curve onto that of the $M_z$ recovery curve,
in Figure 1.7.
Figure 1.6 - T1-w Tissue Contrast.

(A) The recovery of longitudinal magnetization in a tissue, following a 90° RF pulse that has flipped the net magnetization into the orthogonal plane, is shown. The recovery is described by $T_1$, mathematically shown in Equation 1.5. The $T_1$-weighted contrast between different tissues; namely, white (solid) and grey (dashed) matter is shown in (B). Each tissue has a different $T_1$ and by selecting a repetition time (TR) to sample the signal when these values have not recovered to their equilibrium values one is able to weight contrast between them. This contrast is symbolized by the double-edged arrow.
Figure 1.7 - T<sub>2</sub>-w Tissue Contrast.

(A) The decay of transverse magnetization (\(M_{xy}\)) is shown. Here, the T<sub>2</sub> of the tissue examined is a measure of the time needed for the exponential reduction of that signal in the transverse plane, as described in Equation 1.6. The hash marks on the abscissa are meant to indicate that the TR time is often an order of magnitude greater than the TE time. (B) Demonstrates the principle by which contrast between tissues can be generated based on their T<sub>2</sub> properties. The echo time chosen and the contrast effected are indicated by the double arrow between the present tissues.
Finally, proton density weighted images can be generated by long TR and short TE sequences. Essentially, the $T_1$-w and $T_2$-w are both removed. This can be observed looking back at Figure 1.6 at long times. The SI for each tissue is said to be proportional to the proton density of that tissue which can be measured following a 90° RF pulse and giving enough time for full longitudinal relaxation.

An image depicting tissue contrast from a human brain under various weighting strategies is shown in Figure 1.8. Structures of the brain can be discerned under each, corresponding to cerebrospinal fluid (in the ventricles), white and grey matter as well as to the presence of lesions in the blood brain barrier.
Figure 1.8 - Tissue Contrast in the Brain.

This image shows an axial section of the brain of a subject patient under (A) T₁-, (B) T₂-, and (C) proton density (PD) weighting. Contrast of tissues is evident under each pulse regime, however each possesses structurally distinct information. Here, we see the contrast between tissues under different weighting for a patient. (A) We are able to identify regions of fat as bright. Contrast enhancement of a lesion is evident (arrow). (B) The bright regions are areas composed of a higher proportion of water, which highlights the presence of CSF, while in (C) the PD-weighted image shows no abnormalities. The main clinical utility of PD-weighting is to identify abnormalities in neuronal function (5).
1.5 - MR Contrast Agents

1.5.1 - Overview

Confident radiological determination of the presence and extent of disease by MR requires that the biological differences present in tissues be detected and distinguished through changes in nuclear spin. Inherent contrast between tissues can be low, as can be seen in several of the $T_1$ and $T_2$ values found for tissues above, in Table 1.1. This is especially true in the case of particular tissues under normal (healthy) and pathological conditions. The sensitivity of MR to detect these differences is also an issue as noise and other artifacts can contribute to loss of signal and may require prohibitively long acquisition times for imaging. These difficulties render accurate diagnosis based on contrast from MR scans problematic (6).

To remedy the problem of low contrast between tissues and tissue states contrast agents (CA) are often employed. These CA are used to selectively enhance the image contrast in regions of interest by locally affecting the magnetic environment of protons. So-called magnetopharmaceuticals differ from the CA conventionally used in X-ray and X-ray computed tomography (CT), such as iodine, which are directly imaged because of their interference or absorption of electromagnetic energy (7). MR CA function indirectly through their affects on the surrounding $^1$H found in tissues, most often in water (8).

This section will provide an overview of the field of MR CA beginning from their description and categorization. While the focus of the present dissertation is squarely on iron oxide nanoparticulates for MR contrast enhancement, such materials are novel and do not make up the majority of MR CA currently used in the clinic or for research.
purposes. As such, a discussion of the most commonly used CA for MR, gadolinium(III) chelates, will also be provided, in Appendix A, which includes a description of the chemical compounds in clinical and academic use. The present chapter is engaged in a general description of the categorization of MR CA, their mechanism of action and issues associated with their use. The remainder of this section will then be occupied by a synopsis of the field of T_2 and T_2* CA founded on superparamagnetic particles.

1.5.2 - Classification and Measurement of Relaxivity

Contrast agents fall under the broad categories of T_1-weighted (-w) or T_2-w. This is despite the fact that each agent will generally reduce both T_1 and T_2, as their relaxitive effects interact with both transverse and longitudinal processes. Agents are thus classified by the relaxation mechanism that they shorten the most, despite the fact that many agents, such as metal oxides, shorten both significantly (9).

Contrast agents are measured by their efficiency in enhancing proton relaxation. This can be described as the relaxivity of the contrast agent and is defined in Equation 1.8. Here, R_i is the T_1 or T_2 relaxivity corresponding to T_i and C is the concentration of the agent.

\[ R_i = \frac{\Delta C}{\Delta T_i} \]  

Equation 1.8

Thus, relaxivity can be described as the concentration dependent effect of a contrast agent (CA) on proton relaxation.

Relaxivity is most often written in the form of (mM·sec)^{-1} or (mmol/L·sec)^{-1}. These values are determined by a plot of the inverse of T_1 or T_2 time with respect to
concentration of the agent. As from Equation 1.8, the slope of the linear portion of the curve is the relaxivity. Quantitative measurement of relaxivity allows for the comparison of agents and their usefulness under different conditions for diagnostic, in vitro and in vivo systems.
Chapter 1

1.6 - T₂ and T₂* Contrast Agents and Superparamagnetic Iron Oxide

1.6.1 - Introduction

The vast majority of contrast aided clinical scans use gadolinium based agents (10). This type of magnetopharmaceutical is effective at reducing the T₁ recovery time and thus results in brightening in T₁-weighted imaging. While the focus of the work is on T₂* contrast agents, gadolinium based agents are currently the most widely used contrast agents in the clinic. Thus, additional information on the background of and recent advances in the use of gadolinium(III) chelates has been included in Appendix A.

The second class of contrast agents in widespread clinical and research use are T₂ and T₂* agents. There is a wide assortment of different manifestations of these agents utilizing different material and biological strategies to produce localized contrast in vivo. The most common T₂-CA material consists of iron oxide particles on the order of microns down to several nanometers. As these iron oxide materials, particularly nanoparticles (NPs), are the focal point of the research described herein, the discussion of other T₂ and T₂* CA (such as cobalt (11) or ¹⁷Oxygen based CA (12)) will be proscribed.

Using iron oxide in particulate form is common because of several advantageous features of these materials. Iron oxide is a substance found throughout the body and is inherently biocompatible, even at very high concentrations. They provide high change in signal per unit metal and as they are composed of many thousands if not millions of atoms, they overcome the low sensitivity of MR. Further; they can be easily manipulated during and after synthesis resulting in highly derivitized structures possessing application-specific chemical, magnetic and biological properties.
This section will review important features of superparamagnetic iron oxides (SPIO). This discussion will include an overview of the different configurations of particulate iron oxides developed for imaging, the mechanism of action of superparamagnetism-affectted transverse relaxation, the NPs' physical and chemical properties and a discussion of their synthesis. An appraisal of the literature relating the biodistribution and biomedical application of these particles, in their many different forms, will also be given. The final section will focus on their utility for immune cell tracking.

1.6.2 - Terminology

There are many forms of SPIO MR CA in academic and medical use, to the extent that classification of the different manifestations of these agents has become necessary. Categorization of SPIO as defined by their overall hydrated diameter is the most efficient means to that end. However, it should be noted that the literature lacks consistency beyond the groupings below and the nomenclature system often refers to the same particle configurations several different ways. The most egregious are the multiple names for ultrasmall-SPIO (USPIO), as detailed below.

The largest particles, Oral-SPIO have an overall diameter between 300 nm and 3.5 μm and are also referred to as micron-sized iron oxide (MPIO). The more commonly used SPIO formulations are on the nanometer scale. These nanoparticles are defined as particles with one dimension below 100 nm in length. SPIO in this classification include Standard-SPIO (SSPIO) at 60-150 nm, Ultrasmall-SPIO (USPIO) of approximately 5-40 nm and monocristalline iron oxide nanoparticles (MION - a subset of USPIO) range
from 10-30 nm. A prevalent derivatization of MION, consisting of a chemically cross-linked polysaccharide shell, is referred to as CLIO (Cross-Linked Iron Oxide). Molecular imaging of disease generally requires the use of the smaller agents in the category of USPIO. In the literature USPIO is the most common name; however MION, VSOP (very small iron oxide particles) and MNPs (magnetic nanoparticles) are used, among others. SPIO will be used herein to refer to MPIO, SSPIO, MION, CLIO, and USPIO, unless otherwise noted.

Trade names given to SPIO are also often confusing, primarily because the same formulation is often given different pharmaceutical names depending on the specific regional market. Ferumoxsil or AMI-121, an approximately 400 nm Oral-SPIO, is also known as GastroMARK (USA) and Lumirem (EU and Brazil). This agent is approved for use in Europe to distinguish between abdominal tissues and the bowels. Larger Oral-SPIO such as OMP (also known as Abdoscan) has a larger hydrated diameter at 3.5 µm. Ferumoxide or AMI-25 is also known as Feridex IV (USA) and Endorem (EU). This agent is an FDA approved SSPIO, 80-150 nm in diameter, for use in detection and diagnosis of liver metastases. Ferumoxtran or AMI-227 (also known as Sinerem (EU) and Combidx (USA)) is a dextran coated USPIO (of a diameter between 20 and 30 nm) (13) currently in late-phase clinical trials for detection of lymph-node metastases (14, 15). Ferucarbotran, Ferrixan, SHU 555, or Resovist (EU and Japan) is a carboxydextran coated SPIO of approximately 60 nm hydrodynamic diameter approved for use in Europe (16, 17). Table 1.2 contains a concise listing of SPIO, their alternate names, approval status, application and size.
1.6.3 - Alternative SPIO

Overall, the use of the term SPIO in the literature refers to nano- and microparticles as defined above. These magnetopharmaceuticals of superparamagnetic iron oxide are not the only forms of the contrast agents that exist. Ferritin is a well conserved protein complex produced by all eukaryotes for intracellular iron-storage (18). The core of iron surrounded by the protein subunits can be substituted with a superparamagnetic crystal, however the rapid clearance (to the liver and spleen) of these protein-based-SPIO have presented major restraints to their in vivo use (19-21).

In another biologically inspired strategy, iron oxide nanocrystals produced by magnetotactic bacteria have also been exploited for imaging purposes, as an in vivo transgene reporter (22). Here, an adenoviral vector was used to place the nanocrystal manufacturing gene into cells of interest, whereupon subsequent production of SPIO could be visualized using gradient echo sequences. This approach, while certainly novel, has not seen widespread adoption to introduce SPIO biologically.
Table 1.2 - Commercially Available SPIO CA.

A list of SPIO commercially available or in development for clinical use. Updated by Thorek, from (23).

1.6.4 - Iron Oxide Core and Magnetism

Despite the different manifestations of SPIO noted in the preceding section, they typically share two common features. These are a core of iron oxide crystal(s) and an exterior coating material, as depicted in Figure 1.9A. The cores of the particles are composed of inverse spinel iron oxide crystals of magnetite (Fe₃O₄) and/or maghemite (γ-Fe₂O₃). The crystal structures are shown in Figure 1.9B. The cores of many SPIO are composed of multiple crystals or of imperfect crystals, generally dependent on the
synthesis method and conditions. The two magnetic oxides, magnetite and maghemite, have similar crystal structures (lattice parameters of 8.396 and 8.346 Å, respectively) and magnetic properties (24). In bulk, maghemite does have a lower saturation magnetization (saturation refers to a plateau in the magnetic flux density following increasing magnetization force).

This is not a major factor in nanoparticle magnetic properties for two reasons. Firstly, at the magnetic fields that are utilized in MR imaging both forms of bulk materials are invariably saturated. The second and perhaps a more important factor is that at the nanoparticle length scale, the emergence of quantum magnetic effects predominate. This renders the bulk lattice state less crucial in determining particle relaxivity and these features are the real impetus behind the recent interest in nanoparticle and nanoscale technologies. A description of this nanoscale magnetic phenomena (superparamagnetism) follows after a description of the different categorizations of classical magnetic materials.
Figure 1.9 - Schematic of SPIO and Crystal Structure.

(A) Representation of a superparamagnetic iron oxide nanoparticle. The iron oxide core is surrounded by a polymer or polysaccharide coating, either formed during the synthesis of the crystal or after. The surface coating can be chemically modified with functional sites for bioconjugation to biological and chemical items of interest. (B) Crystal structures of a single unit cell for the two predominant SPIO components, magnetite and maghemite, using a ball and stick model. The large orbs are oxygen, denoted as O, the smaller are iron (Fe) in the octahedral and tetrahedral sites. Information for the two crystals were retrieved from the American Mineralogist Crystal Structure Database and images were generated using CrystalMaker®.
Empirically, magnetic materials are characterized by their response to being placed in a magnetic field. The resulting magnetization of the material can take one of several forms and the material is described as being diamagnetic, paramagnetic or ferromagnetic. In the first instance, the orbital electrons in the material are moved to a magnetic dipole moment in the opposite direction of the applied field. In other words, the magnetic susceptibility (magnetization of a material in an applied field) is less than 0. In bulk materials this manifests in repulsion of the material away from the magnetic field. This phenomenon often occurs strongly within materials whose valence electrons are all paired, however all ‘non-magnetic’ materials exhibit some level of diamagnetism. Paramagnetic materials, like the gadolinium(III) ion, have a positive magnetic susceptibility. Thus, the induced magnetization of the material is in alignment with the applied field. However, in the absence of a field, the magnetic dipoles are randomly aligned; in other words they have no remnant magnetization. This is often found in elements with non-zero angular momentum (unpaired valence orbitals) and is found in some metals. A schematic of a paramagnetic material’s magnetic dipole configuration is found in Figure 1.10A.

Ferromagnetism is a phenomenon wherein some materials exhibit spontaneous magnetization in the absence of a magnetic field. The magnetic properties of ferromagnetic materials arise from the alignment of unpaired spins of electrons (magnetic dipoles) in a cooperative fashion. This is observed in some transition metals and their oxides. In a typical bulk ferromagnetic material, magnetic domains are aligned at short range and separated by a transition region called a Bloch wall.
Figure 1.10 – Magnetic Dipole Characterization of Materials.

(A) The random alignment of magnetic dipoles of a paramagnetic material. When exposed to a magnetic field, these spins align and enhance the field. Also shown are the aligned magnetic dipoles of ferromagnetic materials. (B) In a ferromagnetic material of sufficient size to contain crystal sub-domains parallel and antiparallel alignment, a Bloch wall exists to minimize inter-domain surface energy. (C) For a superparamagnetic material, there exists a single magnetic dipole orientation as the crystal size discourages the formation of Bloch walls. This creates a net magnetic dipole for the particle. Edited by Thorek, from (25).
At the nanometer scale, approximately 14 nm and below, the formation of the Bloch walls (magnetic domain boundaries) become thermodynamically unfavorable. The result is single magnetic domain crystals. Magnetite crystals at this scale exhibit very strong paramagnetic behavior, lending them the name superparamagnetic (26, 27). Superparamagnetic materials, like paramagnetic materials, retain no magnetization when removed from the applied field. As such, they are described as having no remnant magnetization. Any cooperative alignment of dipole moments at this nanoscale is overcome by thermal energy at room temperature (28).

The phenomenon of superparamagnetism, first observed in 1938 (29), is now an area of intense research for both magnetopharmaceuticals and data storage (30). The nanoparticles employed for MR image contrast have very high magnetic susceptibilities (much greater than 0). In essence they are saturated in any applied MR field. They function so effectively as $T_2$ and $T_2^*$ CA because of two nanoscale processes: the (1) Néel and (2) Brownian relaxation mechanisms.

The iron oxide crystal is again a single domain particle, but the lattice is anisotropic and thus the NPs magnetization aligns in only so called easy directions. Any non-cubic crystal has a degree of anisotropy energy, determined by Equation 1.9, where $E_a$ is the anisotropy energy of the crystal, $K_a$ is the anisotropy constant and $V$ is the volume of the crystal. Nanoparticulates have high surface-to-volume ratios and so the crystal or particle-core has a high anisotropy constant, but a small volume (31).

$$E_a = K_a V$$  \ \textit{Equation 1.9}

This anisotropy energy determines the Néel relaxation time, $\tau_{N_e}$, described as the time required for the crystal's magnetization to return to equilibrium after a perturbation. This
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relationship is described in Equation 1.10, following the Arrhenius law, where \( \tau_0(E_a) \) is the pre-exponential factor, \( k \) is the Boltzmann and \( T \) is the absolute temperature.

\[
\tau_N = \tau_0(E_a) e^{E_a/kT}
\]  

Equation 1.10

At ambient conditions, \( E_a \ll kT \) (32, 33) and thus the exponential reduces to 1. The Néel time thus depends on the anisotropy energy; again, this value is high for single crystal NPs. As the volume of the crystal decreases, the Néel time decreases and therefore nanometer-sized crystals have very short magnetization angle relaxation times. This results in a magnetization vector that fluctuates randomly in environments of sufficient thermal energy (33, 34).

The impingement of surrounding water molecules upon the nanoscopic entity is also able (at room and physiological temperatures), through transfer of momentum, to randomly translocate and reorient the particle. This latter mechanism is known as Brownian relaxation. This relaxation process is described in Equation 1.11 where \( \tau_B \) is the Brownian relaxation time and \( \eta \) is the damping constant.

\[
\tau_B = \frac{3V\eta}{kT}
\]  

Equation 1.11

The two relaxation mechanisms contribute to a sum relaxation rate, Equation 1.12 (35).

\[
\frac{1}{\tau} = \frac{1}{\tau_N} + \frac{1}{\tau_B}
\]  

Equation 1.12

Under conditions of very fast relaxation, where \( \tau_B \) predominates, the system is always at thermodynamic equilibrium. This behavior is named superparamagnetic (27). An illustration of the two processes found to contribute to the short relaxation times of the
magnetization vector is provided in Figure 1.11A. The NPs are able to recover their equilibrium magnetization faster than any applied impingement upon their magnetic state. The SPIO essentially behave as local magnetic field inhomogeneities leading to rapid dephasing of coherent protons that lie within the SPIO's induced field, as illustrated in Figure 1.11B.

It should be mentioned that nano- and micron sized particles in cells of 5-20 μm can be routinely detected in MR applications using imaging resolutions of 50-100 μm (36, 37). This is a result of the size of hypointensity susceptibility generation by superparamagnetic agents in T₂*-w imaging. Here, the offset phase dispersal in the slice-selection direction produces a triple-band shaped “blooming effect” (38). In phantoms and clinical imaging, the effect produced is much larger than the site of accumulation, producing darkening across a detectable voxel volume (39-41).
Figure 1.11 - Relaxation Mechanisms of Superparamagnetic Crystals.

(A) The two relaxation mechanisms, Néel and Brownian, are schematically described in an applied field in the top and bottom row of single domain crystals, respectively in top and bottom rows. In the Néel phenomenon, the movement of the magnetization vector to some other easy axis and then back to the equilibrium magnetization is described by N. In the Brownian contribution, the rotation of the magnetization results from random movement in water above absolute zero. In (B), we see the local effect of SPIO nanocrystals on surrounding water protons (●→). The effect decreases with an r^6 dependence and thus is highly localized.
SPIO possess both high longitudinal and transverse relaxivities. The majority of uses for iron oxide NPs in the literature are for $T_2^*$-w imaging applications, but they have been used in a variety of applications as $T_1$-w imaging agents. For example, the high $R_1$ properties are exploited in MR angiography protocols where long circulating SPIO configurations are utilized as blood pool agents (42-44).

1.6.5 - SPIO Synthesis

SPIO can be manufactured with distinct sizes and distribution profiles, magnetic character, physicochemical properties and coatings. The most commonly applied methods for producing the NPs are chemical: the co-precipitation and microemulsion techniques. Lesser-utilized routes include ultrasound irradiation (or sonochemcial synthesis) (45), thermal decomposition (46), layer-by-layer deposition (47, 48) and spray and laser pyrolysis (49). These latter methods can provide finer distributions and sizes of NPs, as well as more complex core/shell structures, however they often require advanced equipment and expertise.

In the most widely adopted procedure, that of co-precipitation, ferric and ferrous salts in solution are precipitated by bringing the mixture to high pH, usually through the addition of concentrated base. Equation 1.13 below details the chemical reaction of $Fe_3O_4$ in the aqueous medium.

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$$  \hspace{1cm} \text{Equation 1.13}$$

The base addition precipitation of $Fe_3O_4$ begins at pH 8, at a stoichiometric ratio of 2:1 ($Fe^{3+}/Fe^{2+}$). Absence of an oxidizing environment is required for limitation of side-products as magnetite lacks stability, therefore this chemical route is often run under $N_2$
The partial oxidation of magnetite to maghemite ($\gamma$-Fe$_2$O$_3$) occurs spontaneously, Equation 1.14. This is driven in the presence of oxygen, but may also occur under aqueous conditions through ion transfer particularly at the surface of the as-formed magnetite nuclei (52, 53).

$$Fe_3O_4 + 2H^+ \rightarrow \gamma Fe_2O_3 + Fe^{3+} + H_2O$$  \hspace{1cm} \text{Equation 1.14}

NPs manufactured using the co-precipitation technique offers great flexibility and control over the properties of the final product through relatively straightforward chemical means (54). The advantages of co-precipitation synthesis, in particular large batch volumes, scalability and ease of manufacture, are somewhat offset by wide SPIO size distributions. This is a result of the two steps involved in the technique: (1) nucleation (under inert gas, usually on ice), and (2) growth of the nuclei. The separation of these two steps drives the formation of many small particles followed by the controlled growth of these in the second. Strict size selection, usually by filtration and centrifugation, are subsequently required.

The second most common synthesis method is the microemulsion technique. This route generally achieves greater control of size and shape of the nanoparticles (55). Customarily a water-in-oil approach is pursued that sees ‘nanodroplets’ of aqueous iron salts dispersed and coated in a surfactant-organic solution. The size control over the system is greater for the resulting SPIO as they are precipitated in these pre-sized reverse micelles (again, often through the addition of an alkaline solution) (56, 57). These methods are able to produce populations of particles with very tight distributions. However, a significant tradeoff is that iron precipitates below 5 nm have been found to possess amorphous (non-crystalline) iron oxides (58, 59) and therefore often lack uniform
magnetic properties throughout a population of probes. The yields from this method are also generally smaller in practice than those of the co-precipitation technique.

1.6.6 - SPIO Coating

The use of (typically hydrophilic) surface complexing agents is often required to achieve stability and enhance solubility of iron oxide colloids in physiological conditions, a requirement for use of SPIO in biomedical applications. Repulsive forces between the particles must exist to maintain their stability in solution, as there is some trend towards aggregation from magnetic and surface energy attraction. Bare magnetite particles have a weak interaction with water, leaving the iron oxide surface hydroxyl functionalized. These groups have an isoelectric point at approximately neutral pH (60), meaning that particles will flocculate and precipitate out of biologically relevant solutions unless otherwise coated.

The formation of iron oxides by the co-precipitation technique can either be conducted in water or in the presence of a surface coating materials such as polyethylene glycol (PEG), dextran or polyvinyl alcohol (PVA) (13, 61-63). More recently, chitosan (64, 65) and alginate (66, 67) have been explored as coating polymers because of these polymers' interesting electrostatic and degradation properties. Bare SPIO, which are often produced in a citrate ion solution, can be subsequently coated in small molecules (68-71), silica (72, 73) or polymers (74). Microemulsion produced nanoparticles are often not directly soluble in aqueous solvents, making chemical surface alterations necessary. This can be achieved using surfactants (added either during or after the synthesis).
Beyond magnetically induced aggregation, another benefit of surface complexing agents is to avoid quick opsonization (75). The introduction of bare iron oxide particulate species in vivo is accompanied by rapid opsonization by plasma proteins, resulting in rapid clearance by the reticular endothelial system. This greatly limits the ability of the CA to circulate to bind targets, function as blood pool agents, or distribute effectively into tissues (76). The effect of particular coatings on SPIO pharmacokinetics and biodistribution is a fundamental reason for the wide variety of different materials that have been tested as SPIO exteriors (77, 78). From a size perspective, carbohydrate or polymer-coated SPIO typically vary from ~20 to above 100 nm in hydrodynamic diameter (controlled by synthesis parameters such as the iron-to-polymer ratio) with iron oxide core sizes ranging from ~2 and 15 nm (13).

Surface coatings are also an efficient platform for further modification of the MR CA in that they can be derivatized with different surface chemical groups (79), transfection agents (80), or targeting agents (81). The surfaces can also be used to conjugate the MR probes to other imaging media, for example nuclear and optical moieties to create multi-modal agents (82, 83).

A recent development in SPIO technology has been the investigation of the surface coating effects on the relaxivity of the particles, rather than their biocompatible or biodistribution consequences, as discussed below. The enhanced dephasing of water surrounding the iron oxide core is dependent on that water being within the altered field of the SPIO. With greater thickness coatings it can be realized that there would be less water in the inhomogeneous magnetic region. While the diffusion of water through most surface complexing materials (polymers and polysaccharides) makes computational
modeling more difficult, both simulations and experimental evidence have shown that the thickness of coating affects relaxation properties of the particles (84). Using a fixed iron oxide core size it was found that increasing the thickness of a PEG micellar coating resulted in a dramatic decrease in $R_2$. Interestingly the drop-off in $R_1$ was much less severe, likely due to the rate-limiting step of longer longitudinal water relaxation times.

### 1.6.7 - Biocompatibility and Biodistribution

The broad biocompatibility of SPIO has been a significant reason for its clinical acceptance and basis as a research nanoparticle platform. The metabolism and toxicity of SPIO have been widely investigated since its introduction into the field of medicine (77). Given that iron is an essential element for human beings, and is critical for the delivery of oxygen and the proliferation/survival of all cells, it is no surprise that SPIO can enter the body’s normal iron metabolism cycle following uptake by the reticuloendothelial system (RES) (85). In fact, SPIO were originally developed as a ferritin substitute to treat anemia (86-88).

One concern, however, has been that iron contained in SPIO could enter cells independent of the transferrin pathway (used to closely regulate intracellular iron levels), leading to high levels of non-chelated iron. This is potentially troubling as concentrated iron may produce radical oxygen species. Studies have shown however, that the breakdown of magnetite in cells leads to ferric (rather than ferrous) iron release (89). This iron ion is efficiently chelated by citrate and rendered non-toxic (90).

Extensive research (in vivo and in vitro) has been carried out on the impact of SPIO on cell viability and function. Recent in vitro studies have shown that SPIO are able
to enter the cell (for example, in macrophage (91) and mesenchymal stem cells (92))
through endocytosis and can then either be released back into the extracellular space or
shuttled into low pH environments in lysosomes (92). It is in these compartments where
they breakdown over the course of several days (80, 93). Immortalized fibroblasts, when
incubated with PEG-coated SPIO, showed no discernable changes in cell adhesion
behavior or morphology (87) and the same was reported for T cells labeled with SPIO
(94). Transfection agents such as the HIV-Tat peptide, polyarginine and protamine, as
well as many small molecules, have been conjugated or complexed with SPIO in order to
achieve greater cellular uptake (95). The use of these agents alongside SPIO has also
been found to have minimal cellular toxicity revealing no significant effects on cell
viability (80), clonogenic efficiency (82), biodistribution or immunophenotypic changes,
for example - macrophages are not activated upon uptake (96). Many of these studies also
expose the cells to nanoparticles for long periods of time, up to 3 days, showing little
detriment to cell viability or division kinetics for embryonic (97) and neural stem cells
(98).

While many studies generally assert the indifferent response of cells to the
presence of the probes, further work is required to validate their use in specific
applications and the limitations therein. For example, Feridex IV (Bayer Healthcare
Pharmaceuticals) incubation in culture with human bone marrow-derived mesenchymal
stem cells had no discernible affect on viability, proliferation, adipogenesis or
osteogenesis. However chondrogenesis was found to be inhibited in a dose dependent
manner (99). Working with the same cell line under identical loading conditions, a
polyvinyl pyrrolidone (PVP)-coated iron oxide nanoparticle SPIO formulation was found to possess no phenotype inhibiting effects (100).

Upon intravenous administration of SPIO, anaphylactic-like reactions and hypotension have been noted. Adverse events from SPIO are however usually mild and short in duration (101). The most common complaints included headache, back pain, vasodilation, and hives. Once introduced, the pharmacokinetics of each embodiment of SPIO is dependent primarily on the size and surface characteristics of the agent. The spleen and liver are able to screen SPIO that are approximately 200 nm or above, mainly through mechanical filtration. SPIO below approximately 10 nm are removed through extravasation and renal clearance (102). Agents with a diameter of 30-50 nm have demonstrated longer plasma half-life, particularly when coated in amphiphilic polymers including dextran, PEG and polyvinyl alcohol (PVA) (103). The so-called stealth effect of coatings that inhibit recognition by the RES are thought to act through either avoidance of immune responses that stimulate opsonating proteins or conversely through the absorption of serum proteins that the body recognizes as benign (76).

In the case of targeted SPIO agents, longer circulation times can be equated with an enhanced probability of the probe binding its ligand (104, 105). Longer residency in the blood is also of benefit for non-targeted applications that seek to avert quick clearance by the liver and spleen (106). It should also be noted that large particle size may prevent effective extravasation and reduce diffusion of the agent through the interstitium, important considerations for many SPIO imaging objectives such as lymph node imaging. This is not so much an issue for the imaging of the vascular endothelium (81, 107) or
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Tumor sites possessing leaky vasculature. Here particles have routine access and tend to accumulate through the 'enhanced permeability and retention' effect (108, 109).

Exploitation of this mechanism of passive (non-targeted) accumulation is used to generate contrast in two ways. The first is through SPIO accumulation at the pathological site, thereby darkening it. This has been applied for the detection of cancer and inflammation in many models, including blood-brain-barrier degradation following stroke (110, 111) and neoplasm (112, 113), bone metastases detection (114, 115) and occult lymph node detection (14, 15).

The second passive accumulation strategy is essentially an inverse of that described above. The SPIO may be excluded from accruing in tissue or cells of interest, thus darkening the surrounding region where SPIO access is possible. This effectively results in brightening of the site of interest. This inverse contrast approach is the principal application of SPIO, for the delineation of hepatic metastases. Administered SPIO are found to accumulate in the healthy liver within Kupffer cells, whose primary function is to recycle and accumulate iron from the blood. The absence of Kupffer cells in malignancies allows for SPIO, under T2 and T2*-weighted imaging, to specifically darken the surrounding healthy liver (116-118). This represents a significant improvement over conventional non-contrast enhanced imaging of hepatic tumors. It has been reported that non-contrast-agent enhanced imaging is limited to detection of lesions greater than 3 cm (119), however MR imaging of 1 cm growths is possible with SPIO enhanced approaches (120).
1.7 - Immune Cell Imaging

1.7.1 - Relevance

Directed migration of specific cell types to target organs or tissues is a critical feature of fundamental biological processes. From development through to the maintenance of any complex organism, this trafficking feature is of great scientific interest. With its direct impact on maintaining healthy physical conditions and protecting the body from disease, an area of particularly concentrated research focus has been on the distribution of key cellular players in the immune system.

The immune system is composed of two interacting components; the innate and adaptive. The adaptive immune system is responsible for specific responses to (usually foreign) pathogens, and relies upon T and B cells through processes such as the production of antibodies. This contrasts with the non-specific, but immediate and wide ranging, response mounted by innate immune cells, such as macrophage and mast cells, against a wide range of foreign pathogens. The ability to monitor the selective recruitment, activation, arrival and departure of specific immune cells in homeostatic and disease conditions is a compulsory requirement towards greater knowledge of immune response and function.

Complicating deeper understanding of immune cell responses to pathogenesis and treatment is the fact that imaging of these migratory pathways is inherently difficult. This section will provide a review of the approaches employed to overcome the limitation posed to cellular tracking in the deep tissue of opaque organisms. The alternative
modalities and significant work in cell tracking will be presented. The particular case of utilizing SPIO for MR-based immune cell imaging will then be discussed.

1.7.2 - Tracking Strategies

In order to track specific cell types of interest in vivo it is necessary to deliver contrast media, regardless of modality, directly to that population. There are two general strategies that accomplish this task: 1) ex vivo loading of cells with CA and 2) the in vivo targeting of the population. Each method has unique strengths and weaknesses.

In the first approach, control over the loading of NPs, dyes or genetic constructs into the cells is greater, as in vitro conditions can be optimized for specific cell types. The amount of the CA loading that can be obtained is also much higher as delivery of the agents is direct. This strategy is often also simpler (although exogenous transfection agents may be required) as uptake in unwanted compartments within an organism is not a concern. This means that the addition of supplementary modification such as the addition of a stealth coating (for long circulation) may not be required. The benefit of greater sensitivity upon subsequent implantation (through high levels of accumulated agent) and simpler probe design is balanced by the obligation to first acquire the cells of interest from a biological sample. This may have detrimental effects upon the in vivo function and distribution of these cells. Further, the duration of imaging is limited to the lifetime of the cells (or the residency time of the SPIO within the cells).

The second strategy sees the in vivo application of contrast with some form targetability, usually in the form of peptides or proteins, directed at the cells of interest. These constructs can be repeatedly administered to target the same (or even different) cell
populations of interest. Derivation of the particle with specific ligands is also not always needed, as in specialized applications, for example the investigation of liver metastases using SPIO (121). However, in most applications the non-specific uptake, deposition or accumulation of agent is detrimental to the imaging process. It reduces signal-to-background levels between the population of interest and all non-specifically targeted tissues. This is further complicated by the fact that it is rarely possible to achieve the same loading levels of CA as compared to an ex vivo situation. To overcome this problem of lower signal, interesting tactics are sometimes employed. One example for use in targeting radionuclides has been the two-step system, also called pre-targeting, wherein an antibody-avidin is administered first. Time is given for this first agent to clear before introduction of a biotinylated radiotracer, which has a very high affinity for binding specifically with avidin (122, 123).

1.7.3 - Optical Imaging

Diverse technologies have been used to reveal the spatiotemporal distribution of immune cells in vivo. These include but are not limited to fluorescent and bioluminescent proteins, fluorescent molecules, intravital microscopy, radionuclide-based single-photon and positron emission tomography (SPECT and PET), MR contrast agents and even ultrasound particulate contrast (124). The most commonly used of these options are the optical technologies because of their relative ease of use, and reduced equipment cost.

The major limitation of using light to probe cell distribution within an organism is its limited depth of tissue penetration. It has long been understood that the absorption and autofluorescent properties of tissue is wavelength dependent. This is seen in the
absorption versus wavelength chart in Figure 1.12. Early spectroscopic studies of protein within in intact tissues revealed presence of a near-infrared (NIR) window. This window refers to the much greater NIR transparency of tissue, particularly skin, to light of wavelength in the range 700 - 1300 nm (125). Thus, for effective in vivo studies of cells and cell populations either long wavelength fluorescent molecules or proteins can be effective. Shorter wavelength proteins and dyes can be used, but applications usually involve intravital microscopy. Such techniques are inherently invasive and usually involve surgical exposure of the tissue(s) of interest (126).
Figure 1.12 - Wavelength Dependence of Tissue Absorbance.

The use of longer wavelength dyes for the in vivo imaging of immune cells is explained by the fact that the tissue penetration of such NIR light is much greater than that of higher energy, shorter wavelength light. This feature of greater penetration occurs in the so-called NIR window. The spectral domains of common in vivo imaging molecules and proteins are noted. Figure adapted from (127).
Long wavelength fluorescent sources have been employed in vivo to identify and track immune cell populations. The targeting approach for detection of a cell population centers primarily on organic dye conjugated antibodies. These have been used with some success in visualizing cancers and specific cell types (128, 129). Nanoparticulate forms of optical contrast also exist. Quantum dots (QD) are composed of semiconductor materials with a diameter of several nanometers and are of widespread research focus. They are also found to be phagocytized by activated macrophage when administered intravenously (130). Specific targeting of these NP has not been shown with immune cells.

Ex vivo loading of optical agents for the subsequent in vivo visualization of cells is a common approach for fluorescent imaging. No longer dependent upon applying cell-type specific antibodies (which may be difficult to obtain for the sub-population of interest), lipophilic dyes that bind indiscriminately to the cell membrane have been investigated. In one study, multiple NIR dyes were used in conjunction with specialized laser scanning equipment to concomitantly visualize the whole mouse distribution of activated and naïve primary T cells labeled ex vivo (131). The cells were tracked to the lymph nodes and spleen as well as to tumors implanted in the mouse brain. Labeling of cells with such NIR dyes have been shown to have no effect on T cell proliferation, viability or function (132). Non-targeted QD have also been loaded in cells ex vivo for the in vivo visualization of macrophage and lymphocytes (133).

A concern with the use of any dye is that over long study times, dilution of the dye occurs through cell division. A recent advance in this field has been the development of red (584 nm excitation) and NIR fluorescent protein (684 nm excitation) that can be used to image sites in deep tissue (134, 135). For example transgenic mice, engineered to
express red fluorescent protein in lymphatic tissues have been generated to study T cell migration (136).

Luciferases, luminescent proteins, are also useful for in vivo imaging of cells. Although the light produced is of a short wavelength, the fact that no external excitation source is required means that luciferases can be detected with extremely high sensitivity using specialized imaging systems (137). Only the desired cell types need to be engineered to stably and constitutively express luciferase and the assays are relatively inexpensive. Several transgenic models have used this approach to study human biology and disease (138, 139) detecting as few as 1000 cells in the peritoneal cavity (140).

The majority of in vivo bioluminescent imaging has focused on tumor cells transformed ex vivo to express luciferase and then subsequently implanted. However, immune cells have also been tracked in longitudinal in vivo imaging studies using this strategy. These studies can be divided generally into those that have utilized bioluminescence either to image cells in cancer immunotherapy or to visualize lymphocyte trafficking in basic immune and autoimmune disease models. With respect to the former, the ability of biospecific antibodies to direct cytokine induced killer (CIK) cells towards tumor targets in vivo was rapidly assessed in real time using luciferase (141). The distribution of CIK cells was determined immediately following injection and tracked as they homed to sites of interest 72 hours later (142). Direct imaging of the spread of lymphoma has also been achieved using luciferase. In this work, lymphoma cells were transduced with a fluorescent and bioluminescent construct and implanted into SCID mice. Tumor growth and response to treatment was directly measured (143).
In order to better understand the complex workings of the immune system, visualization of lymphocyte trafficking has been achieved in several transgenic models. In one example, mice have been engineered to only express a luciferase transgene in T cells, enabling highly sensitivity imaging of the migration dynamics of primary lymphocytes (144). This was an effective means to study the antigen initiated clonal expansion and response of CD4$^+$ T cells. Several approaches have also sought to use bioluminescence as a means to better understand the etiology and therapeutic response in autoimmune disorders. In a joint inflammation model of arthritis in a transgenic mouse, T cell hybridomas that bind to collagen peptides were found to target joints (145). Trafficking of this cell population following administration revealed that lymphocytes migrate nonspecifically to sites of inflammation, but are able to linger once exposed to those sites. In an additional study devoted to the early preclinical detection of autoimmunity, a luciferase reporter transgene has been paired to nuclear factor (NF)-κB. Activation by inflammatory responses of NF-κB in a systemic lupus erythematous-like disease model generated bioluminescent signal in organs prior to the gross manifestation of disease or the presence of amplified antibody production (146).

The application of optical methodologies for the detection, tracking and function of immune cells has taken many forms. While several of these techniques and studies have expanded our understanding of cellular function and response in healthy and diseased states, a major limitation to their expanded use is that these agents cannot be used clinically. In order for translational imaging studies to be performed on the distribution of immune cells beyond small animal or in vitro models, the applied cellular and molecular imaging technique must have a clinical counterpart. Current radiological
techniques for cellular and molecular imaging in pre- and clinical study include radionuclide and magnetic resonance modalities.

### 1.7.4 - PET and SPECT Imaging

Radionuclide imaging technologies have been widely employed for visualization of immune cells in healthy and diseased animals. The primary advantage of $\beta^+$- and $\gamma$-ray detection systems is that their sensitivity potentially allows for the detection of single cells. Positron emission tomography (PET) has a detection sensitivity in the pM ($10^{-12}$ M) concentration range; single photon emission computed tomography (SPECT) is slightly less sensitive at $10^{-10}$ M (1). Both imaging modalities rely on the decay of short-lived ($t_{1/2}$ on the order of minutes to hours) or unstable metals. PET isotope materials are produced in expensive, and somewhat rare, cyclotron equipment and decay through positron production that leads to release of two high-energy $\gamma$-rays. These agents have been used for detection by attachment and incorporation in small molecules, conjugated directly to antibodies or mediated through the pre-targeting approach, as described in section 1.7.2.

The most frequently used nuclear imaging agent is the PET radiotracer isotope of Fluorine, $^{18}{\text{F}}$. $^{18}$Fluorodeoxyglucose ($^{18}$FDG), a glucose analogue, is used for clinical detection of cancer cells with upregulated metabolism (147). The agent is extensively used in the clinic for detection and evaluation of neoplasms before and after treatment (148). Uptake at sites of inflammation has long been noted as a source of false positive detection. $^{18}$FDG based PET tests for the detection of atherosclerotic plaque inflammation and ischemic stroke have been completed in both pre- and clinical work (149). The pre-labeling of immune cells (for example white blood cells (150)) with $^{18}$FDG for tracking
to sites of inflammation, rather than localized uptake of small radiopharmaceuticals, has also been successful.

Single-photon imaging using radionuclides such as $^{99m}$Technicium or $^{111}$Indium have been applied clinically for the detection of otherwise occult inflammation (151, 152). The lower sensitivity of SPECT tracers is offset by their imaging system’s increased resolution (versus PET), generally longer half-lives and greater availability to clinicians (chemically rather than cyclotron derived tracers, excepting $^{99m}$Tc). SPECT has been used for immune cell tracking as well, with particular effect in lymphocyte tracking in inflammatory models of bowel disease such as colitis (153, 154).

Radionuclide imaging approaches, while translatable and extremely sensitive for detection and tracking of immune cells, place restrictions on study type and duration. This is primarily due to the shortened window during which signal can be detected as the probes decompose. Further, the resolution afforded by PET and SPECT approaches is limited by quantum effects and detector design. This is made an even greater challenge by the closer proximity of organs at the small animal scale (1, 155). The functional information provided by PET and SPECT can be augmented by pairing these systems with X-ray computed tomographic (CT) systems. While this represents a major advance in both clinical and academic imaging, evidence has been presented that these systems incidentally deliver therapeutic doses of radiation (156, 157). This raises serious health concerns for both subjects and scientists and renders results much more ambiguous (158).
1.7.5 - SPIO and Immune Cell Imaging

MR enables whole body, non-invasive imaging over long time spans, but its low sensitivity requires contrast agents to be present for the imaging of single or groups of cells. Optical and nuclear medical approaches offer their own distinct advantages, however the limited depth penetration (<1 cm for bioluminescence and <10cm for NIR probes) of the former and the ionizing radiation and lack of anatomical detail in the latter impede their translational potential. Cell monitoring studies are difficult with the most common MR CA, gadolinium-ion chelates, which require very high metal concentration to make individual or groups of cells visible. However, it is worth noting that conjugates such as HIV-Tat-DOTA, transferrin-polylysine-DTPA and folate-dendrimer-DTPA have been shown to accumulate in cells and thus could be used to monitor cell migration (159-162). Nevertheless, the intracellular accumulation of toxic gadolinium remains a serious concern.

Biocompatible SPIO particulates offer obvious advantages. SPIO have been used in clinical and academic studies to investigate a variety of biomedical disorders. While the contrast agents have been used in a range of applications (from targeted (105, 163-165) and passive cancer detection (14, 118, 120), diagnostics (166-168), direct (169, 170) and drug therapy (171, 172)), the tracking of cells is a task to which SPIO are particularly adept. First, investigating phagocytic cell types, such as activated macrophage at the sites of inflammation and Kupffer cells in the liver, is made straightforward as these phenotypes readily take up circulating foreign bodies. SPIO have been used to exploit this feature in order to accumulate at and reveal sites of medical
interest; for example, at sites of injured endothelium (91), ischemic stroke (111) or to detect liver cancers (39, 173).

To a considerable degree, the most common cell type detected or tracked using SPIO have been macrophage. The accumulation of contrast agent by this immune cell type renders visualization by MR relatively straightforward, as little additional modification of as-synthesized nanoparticles (or even microparticles) need to be made. Cellular imaging of monocyte/macrophage activity has been employed in a variety of disease models where inflammation plays a critical role. In mice with experimental autoimmune encephalomyelitis (EAE), a model for central nervous system inflammatory disorder multiple sclerosis, administered iron oxide accumulates at cerebral lesions (174). SPIO uptake at these inflammatory sites enables monitoring of progress and staging of disease (175, 176). The pre-clinical application of SPIO for detection of macrophage for the study and detection of organ transplant rejection (177, 178), ischemia (179, 180) and atherosclerosis (181-183) has also been achieved. Further, clinical work has attempted to use the particles to enable better understanding of lesion formation in cerebral models (184) as well as atherosclerosis (185).

Inflammation plays a central role in the pathophysiology of virtually all disorders, and is thus of wide interest. It is also a complicated process involving many cellular players. To this end, cells of many different phenotypes beyond macrophage have been loaded with SPIO ex vivo in order to be followed once implanted. The tracking of inflammatory cells, such as T cells (186), NK cells (187, 188) and dendritic cells (189, 190) has been accomplished. This is a powerful means to gain insight into the locations
of immune cells and their subsequent migrations in response to injury, disease or as therapeutic vehicles (for example engineered T cells or stem-cells (191)).

A focus of recent immunological research, dendritic cells (DCs) are able to stimulate or inhibit immune responses. The important role this cell type plays in marshaling the body’s defenses has made it attractive as a means to augment the native immune response against malignancies (189). A reputed problem with this therapeutic approach has been that the cells do not always localize or migrate to target organs as desired. In vivo imaging of DC migration may substantially aid in the design and administration of dendritic cell-based therapy. Initial studies have shown that when DCs, intended as a cancer vaccine for melanoma, were loaded with SPIO there were no unfavorable effect on phenotype, migration patterns or antigen-presenting functions (192). Further, the utility of tracking as few as $1.5 \times 10^5$ cell with the high anatomical resolution provided by MR has been demonstrated (190). Recently, this work has been translated into the clinic, where trials have established that SPIO enable accurate detection of small populations of DCs administered as therapy to late stage melanoma patients (193).

Natural Killer (NK) cells also hold promise for cell-based therapies as they exhibit high cytotoxic activity against multiple malignancies, while sparing normal cells (188). A genetically modified NK cell line, NK-92-scFv(FRP5)-ζ, has been directed against malignant cells over-expressing HER2/neu (a receptor over-expressed in many cancers, notably breast) (194). MR monitoring of NK cells loaded with SPIO led to distinct and long-lasting recognition of HER2/neu-positive NIH 3T3 mammary tumors in mice (195). Persistent labeling and detection was evident by MR for at least 5 days.
Lymphocytes have been used to detect and treat several classes of malignancies. However, unlike the phagocytic uptake of SPIO by immature DC or macrophage (196), delivery strategies must be employed in order to label T and B cells with SPIO. This can be accomplished several ways including electroporation, lipofection agents and cell permeating peptides (CPPs) (197). CPPs such as HIV-Tat (82, 95), protamine (80) and polyarginine (198) have been used to greatly enhance cellular uptake, allowing for greater loading of SPIO in cells. CLIO-HD, a Tat-conjugated SPIO with high T-lymphocyte uptake, has been used in a system where CD8$^+$ T cells were able to specifically target an antigen expressing tumor upon which it could be clearly delineated by MR imaging (199). Furthermore, T-lymphocytes labeled with CLIO-HD did not lose their ability to kill cells both in vitro or in vivo.
Chapter 1

1.8 - Summary

Cellular and molecular imaging holds great potential for the advanced detection and monitoring of disease in vivo. Magnetic resonance (MR) is a well-suited imaging modality to these ends as it provides high resolution anatomical information throughout an organism. MR cellular tracking approaches require the application of relaxitive contrast agents that alter MR signal in the vicinity of their accumulation.

One form of contrast agent found in widespread academic use are superparamagnetic iron oxide (SPIO) particulates. These materials are often in the form of polymer or polysaccharide coated iron oxide core nanopaticulates. These particles generate contrast by enhancing surrounding $^1\text{H}$ atoms relaxivity, thereby generating local hypointensity on $T_2$ and $T_2^*$-weighted images. The application of SPIO for the task of in vivo tracking of immune cells is expected to provide insight into disease and therapeutic mechanisms.

This thesis aims to identify SPIO synthesis and modification parameters that enable the production of particles with desirable properties for optimal labeling of immune cells. These include control over size, size distribution, magnetic and chemical surface characteristics. The developed contrast agents will then be applied in a series of in vitro and in vivo experiments whose objective it is to track immune cells in rodent disease and therapy models.
1.9 - References


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Chapter 1


Chapter 1


CHAPTER 2. Significance of Thesis and Specific Aims

2.1 - Overview

The use of magnetic resonance (MR) imaging has become an important platform for investigating both basic biological and clinically relevant questions. The state of the art for MR preclinical research is divided along two paths. The first is centered on advancing the capabilities of scanners themselves by employing higher field strengths or pursuing multimodal detection approaches. The compilation of work that is this thesis concerns the second thrust of research - developing, characterizing and utilizing contrast agents (CA). These agents are used to enhance the unfortunately limited endogenous contrast in organisms between cells, tissues or specific sites of interest when visualized by MR.

It has been envisioned that high-resolution longitudinal distinction of immune cells can be of significant utility towards understanding and detection of disease as well as preclinical monitoring of therapeutic efficacy. This chapter first outlines the technical advances of efforts documented in this dissertation as well as the consequent applications that this research will have on the field. This concerns the role of SPIO characteristics for non-phagocytic cell loading and their use for the tracking of and targeting of B cells in
vivo. As well, it involves the identification of sites of neuroinflammation in a rat pain model. The particular goals detailed for each of these sections are then presented.

2.2 - Significance of Research

The ability to image individual or populations of cells in vivo is of great biological and medical interest. The objective of this thesis is to develop and apply biocompatible nanometer-scale contrast agents for immune cell imaging in multiple in vivo and in vitro systems. Superparamagnetic iron oxide (SPIO) nanoparticles are perhaps the most widespread MR contrast in use for molecular and cellular imaging. The fundamental purpose behind the development and application of these agents is to enable earlier and enhanced detection of disease. The use of SPIO specifically for cell tracking and detection of immune cells is a significant component of that effort. This work centered on development and use of SPIO in two medically relevant biological paradigms: monitoring the efficacy of autoimmune therapy and detecting macrophage infiltration at sites of nerve root injury.

2.2.1 - In vivo B Cell Tracking

Systemic lupus erythematosus (SLE) is a chronic, inflammatory autoimmune disorder. Symptoms include fatigue, polyarthritis, pericarditis and dermatitis. Treatment of this disease focuses on immunosupression to prevent organ damage and to combat flare-ups. Among these treatments are non-steroid anti-inflammatory drugs, corticosteroids and, applied in the more serious renal and nervous system manifestations, cytotoxic medicines such as cyclophosphamide (1).
While the pathogenesis of SLE is not fully understood, B cells and B cell dysfunction have been implicated in its etiology (2). Their central role in immune processes has meant that they are a focus of research into the understanding and combat of SLE (3). Recently, monoclonal antibody B cell depletion therapy, which has been effective in treating B cell lymphoma, has been investigated as a therapy for autoimmune diseases including SLE (4). Results from preliminary studies had been promising (5), however under controlled study conditions it has been determined that antibody treatment is ineffective at combating the disease (6-8).

It has been hypothesized that imaging of B cell populations in vivo would be a powerful tool for the in vivo study of B cell response to depletion, particularly within secondary lymphoid organs such as the spleen where long-lived B cells reside and mature (9). We have shown that SPIO can be formulated for the specific task of non-toxic, high uptake in non-phagocytic, non-adherent cells. These SPIO were able to readily label primary murine B cells. Using these engineered particles, coupled with a cell membrane binding near infrared (NIR) dye, primary B cells were tracked using MR and optical techniques following in vivo administration. The data gathered from this work led to an appreciation of the underlying response of cells targeted by antibody depletion treatment and the issues associated with tracking them. Specifically, the work indicates that NIR can be readily used to follow the in vivo migration of cells with organ specificity and a degree of sensitivity such that localization of B cells to the spleen was readily visualized. Further the impact of the MR tracking approach, inherently noisy and seemingly interfering of B cell depletion, may help to mature the still nascent field of nanomaterial application in biology and medicine.
Cervical radiculopathy is defined as a neurological condition characterized by dysfunction of a cervical spinal nerve, the roots of the nerve, or both (10). It presents as pain, often debilitating, in the neck and one arm. This pain is also often accompanied by sensory loss, loss of motor function and diminished reflexes in the dermatome of the affected nerve root (11). The condition is relatively widespread, with studies showing incidence as high as 107 per 100,000 for men and 63.5 per 100,000 for women (12). The frequency of the disorder, coupled with its potential long term and severe impact on quality of life, results in a significant economic impact (13, 14).

The central nervous system (CNS) proximal to the cervical spine, consisting of 7 vertebrae and 8 pairs of cervical nerve roots (in both rodents and humans) is susceptible to injury from a variety of causes. The most common cause of cervical radiculopathy is impingement of the spinal nerve through degenerative changes of the neuroforamen. Radiological evidence of these changes such as spondylosis, foraminal occlusion or disc protrusion is useful in determination of the pain-causing problem and for further decision making in patient management. However, in many cases there is no noticeable neuropathy associated with the report of pain symptoms (12). This presents a significant clinical and radiological challenge.

It has been hypothesized that transient nerve injury may lead to lasting pain without correlating radiological indication of permanent degeneration of vertebral architecture. In a rat model, persistent mechanical allodynia (behavioral sensitivity to otherwise benign stimulus) can be produced by unilateral transient compression of the nerve root (15). The transitory axial loading of the neck that this model seeks to mirror
include examples such as automobile accidents (16) and impact from contact sports (17, 18). Inflammatory cells, such as activated macrophage, have been reported to correlate with pain in experimental models (19-22). Mechanical insult to the nerve root of sufficient compression leads to Wallerian degeneration and remodeling of the neural tissue (23). Macrophages are initially recruited to remove axonal and myelin debris and stimulate nerve growth factor synthesis by Schwann cells. A host of other immune cells are involved including microglia and the recruitment and activation of hematogeneous leukocytes to the site of nerve root compression have been implicated in the etiology of chronic pain resulting in hypersensitivity (24, 25). Further understanding of the development of and the efficacy of treatment for transient nerve root injury would benefit significantly from a means to non-invasively detect the presence of injured nerve roots.

Sequestration of SPIO by macrophages at sites of inflammation in vivo has been demonstrated in a variety of disease models (26-28). This phenomenon has been exploited to image neurologically relevant processes such as cerebral inflammation (29-31), spinal cord (32, 33) and nerve injury (34, 35). No study to date has correlated the uptake and MR detection of SPIO in vivo using a transient nerve root injury with a characterized behavioral response. In the pain producing transient compression model described above, the presence of persistent hypersensitivity has been associated with the infiltration of activated (CD68 expressing) macrophages.

In work described in this thesis, dextran coated SPIO were administered to rats after undergoing a transient nerve root compression to non-invasively detect macrophage infiltration after nerve root injury. It was demonstrated that detection of the site of the injury through accumulation of the SPIO by activated macrophage was possible. We
envision that application of SPIO for the noninvasive, longitudinal imaging of the cellular inflammatory response, correlated to the persistence of pain, will add valuable insight to our knowledge of the underlying dynamics of macrophage infiltration at sites of cervical radiculopathy. Furthermore, it may provide the foundation for enhanced clinical assessment and subsequent treatment of nanoparticle.

2.2.3 - Technical Impact

We envision potential applications of the research described within this thesis will include:

- Study of the effect of size and surface properties on the non-phagocytic cell phenotype uptake of nanoparticulate materials.
- Facilitate the development and refinement of approaches to monitor lymphocytes in vivo.
- Detect the presence of otherwise occult nerve root inflammation resulting from transient compression.
- Identify sites for local administration of analgesic therapy.
- Non-invasively monitor the response to treatment of nerve root injury to treatment.

We envision that the SPIO synthesized, modified and applied in this work will be a powerful and versatile tool for the study of a variety of biomedical problems. In particular they have significant utility in imaging immune cell types of interest in autoimmune and inflammatory processes through their efficient labeling of cells either in or ex vivo.
Molecular and cellular imaging (MCI) is an emergent field concerned with the non-invasive, quantitative, and repetitive imaging of biological processes in living organisms (36). Clinical end-goals include generation of image contrast for the purpose of achieving earlier disease detection, more accurate stratification and monitoring of response to therapy (37). Magnetic resonance (MR) is a powerful non-invasive, whole body imaging modality used in numerous MCI approaches. When used in conjunction with recently developed in vivo contrast agents, MR allows for the concomitant acquisition of anatomically detailed images and molecularly specific information.

The overall purpose of this dissertation was to develop and apply nanometer-scale contrast agents for immune cell imaging in multiple in vivo and in vitro systems. Superparamagnetic iron oxide (SPIO) nanoparticles are T$_2^*$ contrast agents consisting of an iron oxide core surrounded by a polysaccharide coating. In this document we describe the synthesis, modification and evaluation of SPIO for a variety of physical and chemical properties. Further, the interaction of SPIO with cellular systems and animal models was investigated. Initial work focused on development and differentiation of NP populations for non-phagocytic cell uptake. Subsequently, these NPs were applied in vivo to two disease models. The first of these systems concerned the ex vivo loading of contrast agent into B cells for their subsequent tracking in mice with and without B cell depletion therapy with implications for systemic lupus erythematosus (SLE) treatment. The second animal model appraised the ability of systemically injected iron oxide nanoparticles to specifically label sites of neuroinflammation correlated to persistent pain for diagnostic purposes.
2.3.1 - Aim 1. Synthesis, modification and optimization of SPIO probes for cellular labeling

1.1 Synthesis and characterization of iron oxide nanoparticles – Nanoparticle synthesis parameters were varied to develop a library of particles with a wide range of physical and chemical properties. SPIO surfaces were cross-linked and functionalized with amines. The chemical and magnetic properties of these particles were analyzed along with an evaluation of their internal core morphological features.

1.2 Optimize SPIO loading of lymphocytes – We determined a nanoparticle formulation for maximum SPIO loading in cultured lymphocytes, while concurrently avoiding cell toxicity. Flow cytometry, optical and MR techniques were utilized to examine particle uptake. Factors such as particle size, surface properties, loading times and SPIO concentration were isolated in order to determine their respective effects on internalization and viability.

1.3 Evaluate effect of SPIO on B cell activation – SPIO loading conditions leading to activation of primary B cells were assessed. The roles of surface charge, particle diameter, concentration and time of incubation with cells were investigated. Further, B cell activation following SPIO internalization was tested to determine any interfering contrast agent influence on normal B cell function and subsequent depletion.
2.3.2 - Aim 2. B cell tracking in SLE autoimmune therapy model

2.1 Refine imaging of SPIO-loaded B cells – Imaging parameters and a logistical protocol were optimized for the detection of SPIO-loaded and NIR dye labeled B cells injected into mice for both MR and optical (fluorescent) modalities. Following imaging, immunohistological evaluation was utilized to ensure co-localization of SPIO and fluorescent dyes with B cells.

2.2 Image B cell distribution following therapeutic depletion - Anti-CD79 therapeutic depletion of systemically administered SPIO- and near infrared dye-loaded B cells was investigated by MR and optical imaging in mice. Analysis of the dynamics of distribution with and without therapeutic intervention, through loss of local hypointensity for MR and loss of fluorescent signal by optical, was performed.

2.3.3 - Aim 3. Imaging the post-injury dynamics of macrophage infiltration

3.1 Optimize MR sequence and injury localization – Localization of transient nerve root compression was carried out. Initially this involved posthumous subjects to resolve animal handling during imaging and MR sequence issues. Living subjects were then introduced to determine feasibility of imaging protocol. Delivery and optimization of SPIO contrast by MR was also performed by varying scanning parameters for sequence configuration.
3.2 Imaging transient nerve root injury – Visualization of macrophage infiltration at the injury was evaluated across injured and sham subjects at time points prior to and following administration of contrast. Histological analysis of excised spinal cord and nerve root were used to verify SPIO accumulation at sites of macrophage infiltration.
Chapter 2

2.3 - References


CHAPTER 3. Synthesis, Modification and Characterization of SPIO

3.1 - Overview

3.1.1 - Preface

A promising new direction for contrast-enhanced magnetic resonance (MR) imaging involves tracking the migration and biodistribution of superparamagnetic iron oxide (SPIO)-labeled cells in vivo. Despite the fact that a number of cell labeling studies have been performed with SPIO particles of differing size and surface charge, it remains unclear which SPIO configuration provides optimal contrast in cells of interest. One of the central goals of this thesis was to accomplish immune cell tracking in multiple disease models. To that end, an engineering approach to manufacture and characterize nano- and microparticles was undertaken. The initial aim was to develop good manufacturing processes for a SPIO formulation that enabled efficient delivery of contrast agent to cells in an ex vivo loading regime.

The present and following three chapters are connected components of a single research thread. The overall goal was for the use of SPIO nanoparticulates to enable imaging of lymphocyte trafficking in vivo. SPIO were developed and their interaction with cells investigated for that purpose (see Chapter 4). The specific application was to
track B cells in the mouse and monitor their in vivo distribution following administration of a B cell depleting therapy (see Chapter 5).

It can be seen that there are two general strategies for SPIO-enabled visualization of a population of cells; 1) loading the cells ex vivo with SPIO and 2) specific targeting of cells of interest in the organism. In the former, higher sensitivity for cell tracking is afforded as the ex vivo loading of cells with SPIO is high. Thus high contrast per cell can be achieved. However, cells must first be isolated.

In the latter system, cells need not be removed or exposed to an ex vivo environment. Targeting of the SPIO to cells of interest would involve their modification with a ligand for cell specificity, for example antibodies, and enable repeated administration of such an agent (see Chapter 6). As a result, loading efficiency is lower following this approach and there is the likelihood of greater background uptake (for example by the reticuloendothelial system).

The present chapter is concerned with the basic processes of manufacture and characterization of the nanoparticles used throughout this thesis. Production of particles with desirable features (including size, distribution, surface and magnetic properties). The following chapter utilizes these particles to address the problem of labeling non-phagocytic cells, such as T and B cells. Subsequently, the application of this work was undertaken in an animal model for the non-invasive spatiotemporal visualization of murine B cells loaded ex vivo and injected into host mice.
3.1.2 - Abstract

The high efficiency delivery of SPIO to cell types that do not actively internalize foreign material has presented a significant problem within the field of cell tracking. In phagocytic cell types, such as macrophage, achievement of loading levels on the order of tens of picograms of iron per cell is not difficult. Facile incubation in culture for a period of several hours is all that is required (1). For non-phagocytic cells the intracellular loading levels needed for MR visualization are most often realized through application of potentially toxic biological or chemical modifications to the SPIO or the use of disruptive treatment of the cells (such as electroporation) (2).

Despite the size of this body of work, it remains unclear what configuration of SPIO provides optimal contrast for non-phagocytic cell types. The presence of contradictory findings, elaborated upon below, can be attributed to a significant degree to variability and imprecise control over particle size or surface charge and the limited number of particle configurations examined in any given study. In the present chapter, we have manipulated the synthesis procedure for SPIO nanoparticulates as well as systematically characterized these and commercially available NP. Dextran coated iron oxide particles in a continuum of sizes were generated with low polydispersity. These materials were subsequently cross-linked for stability and functionalized with surface amine groups. Control of the density of these amines was demonstrated. The core structure of the particles was also evaluated and surprisingly revealed that the ultrasmall and standard SPIO (USPIO and SSPIO, respectively) were multi-core in nature.
3.2 - Background

The growing field of nanoparticle (NP) research includes a plethora of different types of materials. These include (but are not limited to) particles composed of metals and metal oxides (such as gold, silver and SPIO), lipid and polymeric micelles or vesicles and protein complexes. Further, many are composites of several classes of materials, notably the polysaccharide and metal-oxide SPIO particles used throughout this dissertation. Characterization of these materials involves considerable effort as it is their nanoscopic physical and chemical features that drive interest in their design, fabrication, modification and application.

Traditional materials characterization technologies that rely on sampling bulk properties, while still important, are just the beginning of the descriptive route when working with nanoparticulate matter. This section seeks to provide the reader with an understanding of the field of materials characterization as it applies to NPs and specifically SPIO. This is significant because material properties optimization and manipulation are substantial elements of this thesis as we investigate the application of SPIO to ex vivo and in vivo cellular systems.

3.2.1 - Size and Mass Measurement

Techniques that are applied widely in the field of colloidal nanoscale research begin with size measurement. There are several methods to characterize a NP's size, most notably atomic force microscopy (AFM), electron microscopy (EM) and dynamic light scattering (DLS). Other solutions based methods that are less common include
sedimentation velocity and size exclusion chromatography. Conceptually the simplest method, AFM sees the deposition of a NP solution to an ultra-flat surface (usually cleaved mica), which is then rastered using micron-sized cantilevered-beam with an atomically fine (usually silica) tip. In many ways the system can be thought of as the needle of a phonograph that is sensitive to the pits of a record. A laser beam is directed to the cantilevered-beam. When moved across the surface containing the deposited particles, interaction of the atomic tip with the deposited material deflects the beam which is measured by changes in the laser reflection (3). The result is non-destructive topology of the materials on the surface, measured in either air or water, that can extend all the way to atomic (attometer) resolution (4).

With one notable exception (the measurement of SPIO interaction with telomerase as an enzymatic nanosensor (5)) SPIO are not often measured using AFM. Instead DLS and transmission EM (TEM) techniques are often employed. The reason for this is that this NP configuration of polymer-coated iron oxide core is both stable in solution and possesses an electron dense core. This means that additional information, generally in higher throughput, can be obtained regarding their size distributions than is possible with AFM.

DLS probes particulates on the 1 - 1000 nm scale. NP in solution are placed in a cuvette and irradiated with the polarized light of a laser. The vast majority of light passes through the sample, however some is scattered to a detector. The intensity of scattered light, detected at some fixed angle (usually 90° from the incident laser) is dependent on the diffusive motion of the particles in the solution (6). An autocorrelation function applied to the scattered light intensity of the nanoparticles reveals the Brownian motion
(diffusivity) of the particles, which can be used to calculate their size (following the Einstein-Stokes relationship). This autocorrelation of the intensity is why the technique is sometimes referred to as photon correlation spectroscopy. The autocorrelation function employed influences the final results. Most assume the presence of monodisperse spherical particles. While that is not often a true representation of the physical reality, comparison of several sizing models, standards and verification by other methods (for example TEM) has shown them to be quite accurate (7). Sample preparation for DLS is extremely easy, and requires only that particles remain in suspension throughout the measurement (often on the order of several minutes). DLS measures the hydrated particulate diameter as the sample is in solution. This is relevant for SPIO characterization because it reports the composite particles' inclusive size.

Transmission electron microscopy (TEM) provides some of the most detailed views of matter. Passing a focused high voltage beam of electrons through a sample is the basis for this technique. Sample preparation is onerous and time consuming, requiring particulate samples be carefully dried onto specialized 10 mm diameter grids. Ideally the sample is dried into a monolayer, as the working distance of this microscopic technique is limited to several hundred nanometers. The grid upon which they are placed is usually a metal mesh (often copper, titanium or nickel) that is then sparsely coated with thin layers of carbon or silicon. The presence of a particle that absorbs much of directed electron beam on this thin (minimally absorbing) layer allows for the distinction and measurement of the particle size. In the case of the many NP materials composed of or containing electron-transmitting substances (usually carbon-rich materials such as polymers, lipids or polysaccharides) absorbent material such as gold or carbon must be coated onto the
particles. In the case of non-metal coated SPIO this is actually an advantage for characterization purposes. For example, with the NPs synthesized and applied in this thesis, the electron-transparent dextran is generally not detectable rendering the iron oxide cores visible. This enables the direct measurement of the magnetic cores of the particles. When combined with data gathered from DLS measurements, information about the core composition and size, the entire diameter and the thickness of coating can be compiled.

These methods give information regarding particle dimensions, however it should be noted that none give a direct measurement of the mass of the particles. Acidic degradation of iron oxide particles can be used to spectrophotometrically assess the iron content in a solution, but without an understanding of the mass per particle, this gives no correlating information to the particle concentration in solution. Determination of core size from TEM can be used to compute the mass per particle (assuming an appropriate atomic packing factor and geometry). This estimation method is however limited to a small sample size (as cores must be measured one-by-one). Other methods exist for mass quantification of particulate samples. One is to take a quantity of sample and dry it at high temperature. The recovery of iron residue from the sample can be assessed by weight to estimate the iron per particle. This method depends on the assumption that all iron is present as iron oxide that the DLS measured volume is monodisperse and is heavily dependent on assumptions regarding the mass of any coating material. A third method for the determination of core size relies on the viscosity of a solution that contains suspended particles. A relationship exists between the viscosity of a dilute solution of nanoparticles and the volume fraction of particles in that solution.
Determination of this volume fraction of particles in solution, again coupled with DLS information regarding the entire particle diameter (and therefore volume), enables calculation of the number of particles in solution. In turn, spectrophotometric analysis of a solution of nanoparticles provides the iron concentration, which when divided by the number of particles of an equal volume provides an estimation of core size (9). The assumptions employed in this approach are that the particles are spherical and that the contribution of mass of iron greatly outweighs that of the coating material.

3.2.2 - Chemical Surface Profiling

Equally important as the size and mass of NPs for their in vitro and in vivo application is their surface makeup. There exist several methods to assess the physico-chemical properties on particle surfaces. Fourier transform infrared spectroscopy is a relatively inexpensive technique that relies on long wavelength light interference by dried or aqueous materials. Atomic bonds absorb this low energy electromagnetic radiation in specific spectral domains. This method is only somewhat quantitative but has been used to determine the presence or absence of specific chemical species on iron oxide NP surfaces (10, 11).

Spectrophotometric methods (UV/Vis) have been widely used to determine nanoparticle surface functionalization. Quantitative surface group assessment can be accomplished in several ways: 1) through the direct detection of small molecules on particles surfaces through specific absorption bands, for example to detect folate (10) or 2) the reaction of surface groups with secondary absorbent (or fluorescent molecules) (12). An example of the latter that has been used with great success to determine the
amine surface content of SPIO has been the use of the thiol cleavable agent succinimidyl 3-(2-pyridyldithio)propionate (SPDP). SPDP can be used to react with surface amines which, following cleavage and purification, yields the 2-pyridinethione chromophore. This molecule absorbs 343 nm light once cleaved allowing for determination of its quantity in solution. SPIO are reacted with SPDP and the excess chemical is removed from solution. The SPDP is then cleaved and the 2-pyridinethione is quantified. The concentration of this molecule divided by the concentration of particles in solution is a sensitive estimate of the number of amines per particle (13, 14).

The catalog of SPIO surface compositions is nearly endless. Different polymer or metal coating materials have been used that present, or are subsequently modified to possess, nearly every conceivable functional group. Only a small subset of these, including amines, carboxyls, thiols and azides are discussed in this thesis.

3.2.3 - Magnetic Properties

The advantages of cellular and molecular imaging using MR are considerable. It allows for determination of the presence and in some cases the state of chemical, biological and cellular items of interest. This information is conveyed with high spatiotemporal distinction non-invasively. The use of contrast agents are in many cases required for this imaging. The characterization of relaxivity of materials, a description of their concentration dependent affect on local magnetic field, was introduced in section 1.5. Material magnetic properties may be assessed either in dedicated instruments (such as superconducting quantum interference magnetometers) or directly in MR imaging systems. Dilutions of sample are prepared and read, and the requisite curves are
computed. The linear portion of the $1/T_1$ of $1/T_2$ versus iron concentration yields the $R_1$ or $R_2$ measurement, respectively.

Another characteristic of magnetic materials is their saturation magnetization. This refers to the magnetic field strength at which no greater enhancement of material magnetic field can be generated. This is a useful defining characteristic of magnetic materials for applications such as electronics and specifically data information storage. Assessment of NP magnetization hysteresis curves and saturation values are often drawn from a superconducting quantum interference device (SQUID) or vibrating sample magnetometry (15). Extremely accurate measurements, even of single particles using SQUID can be made (16). However, the field strengths of MR imaging are of sufficient strength (approximately six orders of magnitude greater than the earth's magnetic field) that all magnetic materials are saturated.
3.3 - Materials and Methods

3.3.1 - Nanoparticle Synthesis

Three different formulations of dextran-coated superparamagnetic iron oxide nanoparticles were prepared using the co-precipitation method (17). All three formulations were prepared following the same procedure, as described below, with the only difference being the amount of FeCl$_2$ and FeCl$_3$ added. Specifically, 25 g of dextran T10 (GE Healthcare; Piscataway, NJ) was dissolved in 50 mL of dH$_2$O and heated to 80˚C for 1 hour. The solution was allowed to return to room temperature and continued to mix overnight. Subsequently, the dextran was cooled to 4˚C on ice and degassed with N$_2$ for 1 hour. FeCl$_2$ (0.7313 g, 1.5 g, or 2.2 g) and FeCl$_3$ (1.97 g, 4 g, or 6 g, respectively) were each rapidly dissolved in 12.5 mL of degassed dH$_2$O and kept on ice for approximately 10 minutes. The iron solutions were added to the dextran simultaneously and allowed to mix for 30 minutes. Keeping this mixing solution at 4˚C, 15 mL of ammonium hydroxide was added. The resulting black viscous solution was then heated to 90˚C for 1 hour then cooled overnight, followed by ultracentrifugation at 20k rcf for 30 minutes. Pellets were discarded and the supernatant was continually diafiltered using a 100 kDa MWCO cartridge (GE Healthcare) on a peristaltic pump (E323, Watson Marlowe Bredel; Wilmington, MA). The particles were exchanged into 0.02 M citrate, 0.15 M sodium chloride buffer until all unreacted products had been removed. Aminated silica-coated iron oxide micro-particles were purchased from Bioclone Inc. (San Diego, CA). Amine functionalized styrene-copolymer coated iron oxide particles (Adembeads) were purchased from Ademtech SA (Pessac, France).
3.3.2 - Modification of Particles

Amination and cross-linking of the coating on the dextran-SPIO was accomplished through reaction of the SPIO with 25% 10 M NaOH and 33% epichlorohydrin (18). After mixing for 24 hours, the particles were transferred into regenerated cellulose dialysis membranes (8-14 kDa MWCO, 50 mm flat width) and dialyzed against 0.02 M citrate for 10 minutes. After transfer back into glassware, additional ammonium hydroxide was added to the solution, bringing the volume fraction to 25% ammonium hydroxide, and the reaction was allowed to proceed for another 24 hours. The particles were then exhaustively purified via diafiltration. The resulting particles were amine functionalized cross-linked iron oxide.

All SPIO particles were labeled with FITC at a FITC-to-iron molar ratio of 19.2:1. FITC (389.4 g/mol) was dissolved in dimethylformamide and added (10% v/v) to SPIO. SPIO concentration was determined spectrophotometrically (following 6 M HCl decomposition in the presence of 3% H₂O₂) against a standard curve to yield molar iron values. FITC was reacted with particles for 4 hours followed by two rounds of gel purification, once on a NAP-5 column and then on a PD10 column (GE Healthcare), both equilibrated with PBS. The FITC-labeled SPIO were subsequently reacted with various volumes of glycidol (0.01% to 50%) to produce populations of particles with different surface amine content. The particles were cleaned of excess glycidol through repeated precipitation in isopropanol and resuspension in phosphate buffered saline (PBS). Amine-blocking was also attempted with particles 200 nm and greater, however this modification impelled immediate particle insolubility.
3.3.3 - Overall and Core Size Measurements

The hydrodynamic diameter of the dextran-coated and commercial iron oxide particles was measured using a Zetasizer Nano-z (Malvern Instruments, Malvern, UK) through dynamic light scattering (DLS). The dextran-coated SPIO particles were diluted in PBS to a concentration of approximately 0.5 mg/mL and read in triplicate. The commercial particle diameters were read in the same manner, but only after undergoing three washes by precipitation in the presence of a strong magnet and resuspension in PBS. The values reported for all samples are the intensity peak values.

Transmission electron micrographs of all iron oxide particles were taken using a JEOL 2010 at 200 kV. Samples were prepared for imaging by evaporating the particles onto a carbon-coated copper grid (Holey carbon - mesh 200, Structure Probe Inc., West Chester, PA). Salt was removed from all of the samples prior to evaporation by exchanging the particles into dH₂O. Images of particle cores were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Since many of the particles were found to be composed of a cluster of multiple iron oxide cores, the average diameter of each core and the average number of cores per particle were determined. Assuming each core to be spherical, the amount of iron per particle type was determined from the aggregate core volume.
3.3.4 - Magnetic Characterization Assays

The longitudinal ($R_1$) and transverse ($R_2$) relaxivity of each particle was calculated as the slope of the curves $1/T_1$ and $1/T_2$ against iron concentration, respectively. $T_1$ and $T_2$ relaxation times were determined using a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz at 40˚ C; Bruker Optics Ltd, Billerica, MA). $T_1$ measurements were performed by collecting 12 data points from 5.0 to 1000 msec with a total measurement duration of 1.49 minutes. $T_2$ measurements were made using $\tau = 1.5$ msec and 2 dummy echoes, and fitted assuming monoexponential decay.

3.3.5 - Chemical Assessment

The number of amines per particle was determined following the general procedure described by Zhao et al. (14). Briefly, iron oxide particles at a concentration of 2 mg Fe/mL were reacted with excess N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Calbiochem, San Diego, CA) for 4 hours. SPIO were washed of excess SPDP through repeated precipitation in isopropanol and resuspension in PBS. The particles were then run through a 50 kDa MWCO centrifugal filter (YM-50, Millipore, Billerica, MA) either with or without the addition of disulfide cleavage agent tris(2-carboxyethyl)phosphine (TCEP). The difference of the absorbance of these two samples at 343 nm was used to determine the concentration of 2-pyridinethione in the filter flow. Adjusting for dilution, the number of amines per particle was determined, assuming one 100% conjugation of one SPDP per amine. The stability of cross-linking of dextran was measured by heating the particles in a dry bath to 80˚ C for several days. Samples were run through a 50 kDa MWCO filter at different time points. The presence of freed
dextran was colorimetrically revealed using the phenol:H$_2$SO$_4$ sugar method (19). The concentration of flow-through dextran was compared to a standard curve of dextran in dH$_2$O.
3.4 - Results and Discussion

3.4.1 - Particle Synthesis and Modification

Three different formulations of dextran-coated superparamagnetic iron oxide nanoparticles were prepared via co-precipitation. All three syntheses utilized a ratio of approximately 3 ferrous to ferric iron chloride; however, the total amount of iron was increased by whole numbers, i.e. 2x and 3x iron respectively. This deviation in the amount of iron present during synthesis allowed for the manufacture of SPIO with a range of different sizes and properties.

A wide range of synthesis conditions were assessed in order to determine a working procedure that enabled repeatable production of stable, superparamagnetic, high yield nanoparticles. Parameters in the synthesis that were altered in order to determine their role in nanometer SPIO production included; 1) reaction temperature, 2) atmosphere, 3) molar ratio of iron salts, 4) type of base, 5) rate of base addition, 6) stirring speed, 7) post-precipitation heating time, and 8) ratio of iron to complexing agent. The complete characterization of the SPIO was not attempted until high quality nanomaterials were first produced, and thus the results of the initial engineering of the SPIO were predominantly observational rather than quantitative.

Several of the reaction components were deemed to be critical towards production of useable magnetopharmaceutical. First, an inert atmosphere was found to be required to produce magnetite and maghemite. This observation was based on the color of the complexes formed during reactions conducted at both 4˚ C and 25˚ C in the presence of polymer (dextran) or surface complexing ions (such as citrate). The formation of a red or
orange complex followed by detection of large iron precipitates was noted when reactions were initiated in air. This color is indicative of the presence of iron oxyhydroxides that are diamagnetic (20, 21). Reaction under N\textsubscript{2} was found to prevent the oxidation of material during the synthesis, as reported generally in the literature (22).

Critical parameters were the temperature of the reaction and the rate of base addition. It was assessed that these two elements of the procedure were intimately related. The reduction of the thermal energy present in the aqueous reaction was mediated through the presence of an ice bath and the addition of base at a slow rate. The slow addition of base (regardless of type, sodium hydroxide or ammonia) and the effective dissipation of heat produced from acid/base titration was critical. Together, they controlled the heterogeneous nucleation of the iron oxides (in the presence of polymer) delaying the ripening of particles (23, 24). This is illustrated schematically in Figure 3.1.

This finding may also apply to the observation that larger reaction volumes were not effective for production of particles with sufficient polydispersity. Although only a limited number of larger (250 mL) reaction batches were attempted, it is hypothesized that the lack of a thermal jacket or more specialized cooling equipment resulted in inadequate temperature control.
Controlled synthesis of particles, mediated primarily by presence of solute and temperature, allowed for the segregation of the nucleation and growth phases of the nanoparticles. During the nucleation phase (at 4°C) the iron ions do not spontaneously form stable nuclei of NP as the reaction conditions are conducted below saturation concentration of metal ions. However, upon the addition of base there is the heterogeneous nucleation of NP on dextran polysaccharides. By keeping the thermal energy in the mixture minimal, while still allowing for efficient mixing, a very large number of small particles are formed. These are then grown into larger particles with the application of heat, likely through a ripening process, to produce fairly uniform particles. Adapted from Sugimoto et al., (25).

Figure 3.1 - Nucleation and Growth of Nanoparticles.
Post-precipitation heating, at levels of between 80˚ and 100˚ C was found to be required for contrast applicable magnetic properties. The reason for this is not exactly known, as the temperatures used here are far below that required for the conversion of iron oxides to maghemite and magnetite (24). It is likely that high relaxitive properties result solely from growth of pre-nanoparticle nuclei into actual nanoparticles, however it is also possible that relatively low temperature (90˚ C) heating of an iron oxide nanomaterial may provide sufficient thermal energy for a crystal structure phase change. It should be noted that further heating for greater than the (often used) 1 hour did not produce larger SPIO. This may be because the formation of particles of a defined size are sterically stable. Therefore, agglomeration at a SPIO-size plateau is reached after some size apparently dependent on initial synthesis parameters.

When altered, several parameters provided little observable difference in the products of synthesis. The concentration of the iron salts was found to be of little importance and several reactions were run with lower concentrations (and corresponding lower concentration of dextran) that resulted simply in production of less final product. It had been hoped that lowering the concentration (dilution) of the initial material would result in smaller or more monodisperse particles. Further, despite observations in the literature that change in the molar ratio of the salts, for example a 3:1, 2:1, 1:1 or 1:2 ratio of Fe\(^{2+}\):Fe\(^{3+}\), having an effect on the final product, all apparently yielded nanoparticles.

Another factor of little significance was stirring speed. Effort was expended to determine if stirring speed of the solution could result in, again, smaller or more monodisperse particles. The hypothesis here was that better mixing conditions would ensure that minimal heat would be evolved from acid-base titration and that greater
mixing in general would provide a more homogeneous reaction. Difficulties were encountered using external (non-magnetic stir bar) top down stirrers. While faster mixing was attainable, it was at the cost of a less well contained inert atmosphere. Results were not highly consistent between batches using top down mechanical stirring apparatus, and there was little apparent difference between using magnetic stir bars.

Besides the rate of base addition, the parameter that had the greatest effect on the geometric and magnetic properties of the nanoparticles was the molar ratio of the complexing agent to the iron salts. A wide variety of concentrations of both of these components were used (keeping the $\text{Fe}^{2+}:\text{Fe}^{3+}$ ratio at 3:1). Increasing the ratio by means of more dextran relative to a fixed iron amount quickly resulted in a viscous reaction volume that inefficiently mixed. Thus, a fixed amount of dextran was used with varying amounts of iron salts. It was found, as discussed in detail below, that increasing SPIO size and magnetic properties resulted from increasing the ratio of iron to dextran. When the total amount of iron was increased beyond 8.2 g, the co-precipitation solution became extremely viscous and yielded highly dispersed aggregates that precipitated out of solution.

Core analysis of the particles was carried out in order to study the internal structure of the variously synthesized particles and to correlate these features to bulk colloidal properties. It was revealed by characterization that individual component cores between all particles were similar and it was the number of cores that differed. This suggests that the phenomenon leading to greater size and relaxivity was not greater size of particle nuclei but greater agglomeration during the growth phase.
Finally, the chemical surface features were analyzed. It was found that modification of the particles, due to the excess of reactants used, was a highly consistent process in all of the cross-linking, functionalizing and blocking procedures. At the time that the cross-linking and amination procedures were undertaken here there was no defined literature on the subject. Subsequently, an excellent source method, from Pittet, et al. (18), has been published that closely mirrored the finalized protocol used here. The only major difference occurs during the transition step just prior to adding ammonia for the amine-functionalization of the particles. We found that a brief dialysis step, followed by addition of fresh NaOH, allowed for a more consistent product. We can only speculate that the result of this quick wash removed some (primarily spent) epichlorohydrin from the volume and enable more efficient amination with the diamine ammonia. The glycidol blocking of amines was also conducted in very high initial excess conditions. The SPIO were not greatly affected in even 50% volume of glycidol (they remained in solution; a rare phenomenon when mixing the particles with non-aqueous media at that volume ratio) allowing for conversion of the required ratio of amines.

3.4.2 - Shell and Core Characterization

Characterization of the SPIO size was accomplished using optical scattering and TEM measurements. These techniques determined the overall diameter and core diameter of the particles, respectively. The DLS of the SPIO, following cross-linking and amination of the dextran coating, indicated an average hydrodynamic diameter of 33.4, 53.5, and 107 nm respectively, with the larger nanoparticles corresponding to syntheses that utilized greater iron to dextran ratio. Nanoparticles ranging from 200 nm to 1 μm in
diameter were acquired from commercial sources in order to supplement the as-
synthesized particles (which were limited in size beyond an initial iron concentration due
to viscosity of the solution becoming too great). Specifically, superparamagnetic iron
oxide particles of 200 and 300 nm diameter with an amine functionalized styrene-
copolymer coating (Amino-Adembeads) were purchased from Ademtech, while amine
functionalized silica coated 1μm diameter particles were purchased from Bioclone. This
allowed particle sizes across nearly three orders of magnitude to be compared.

The particle sizes as determined by DLS, as peak intensity values, are compared in Figure 3.2. The 33.4 nm, 53.5 nm and 107 nm dextran-coated SPIO samples were fully soluble at physiological conditions. Conversely, it was found that the large size of the 289 nm and 1430 nm particles led to precipitation.
Figure 3.2 - Hydrodynamic diameter of SPIO.

The hydrodynamic diameter of SPIO particles was determined by DLS. Intensity measurements are reported and the peak intensity is provided for each distribution.
Analysis of the iron oxide core size and structure of the magnetic particles was conducted using TEM. Representative micrographs are shown in Figure 3.3. Aggregation of particles in salt free solution was a problem during TEM sample preparation; however, reduction in sample concentration allowed for imaging of discretely distributed particles after solvent exchange to water. Iron cores were easily distinguished from carbon coated copper grids, while dextran and styrene copolymer were not visible because of their low electron density. The edge of the holey grids is evident in image panels D, E and F.

An interesting and unexpected feature of the dextran-coated nanoparticles was that each particle consists of a cluster of one or more iron oxide cores. Furthermore, each core was found to be approximately equal in size. Specifically, the distribution of cores is centered at approximately 6 nm for all three dextran-coated nanoparticles (Figure 3.4); however, the average number of cores per particle increases with overall hydrodynamic diameter. In contrast, the larger 207 nm and 289 nm styrene copolymer-coated particles exhibited a single large spherical iron oxide core, while the 1.43 \( \mu \text{m} \) silica-coated particles exhibited an amorphous iron oxide core of no discrete size or shape.

It had been initially assumed that a larger particle diameter as determined from solution based DLS would contain a larger single core. It had been predicted that the iron cores would be imperfect magnetite or maghemite crystals, due to the fact that the crystals were produced using an agitated aqueous solution reaction approach. Discovery of the multicomponent core indicates that under the reaction conditions used, nuclei size of approximately 6 nm is produced independent of the ratio of iron ions to dextran. This ratio affects the ultimate size and core composition by altering the number of these nuclei.
per particle. Core diameters from a large sample of TEM measurements were used to compute the average diameter as well as undertake a determination of the mass per particle. Using a packing factor of 0.68 and assuming a spherical geometry (9), the number of iron atoms per core and particle is given in Table 3.1.
High magnification transmission electron microscopy images of the iron oxide particles were obtained with a JEOL 2010 operating at 200 kV. Structure analysis revealed the multiple core nature of the (A) 33.4 nm, (B) 53.5 nm and (C) 107 nm dextran-coated SPIO. Larger particles were composed of single cores; (D) 207 nm, (E) 289 nm and (F) 1430 nm. All scale bars are 50 nm, excluding (F) 1 μm.

Figure 3.3 - TEM of SPIO Cores.
Figure 3.4 - Size Distribution of SPIO Core Diameters.

TEM measurements of the SPIO core diameter for (A) 33.4 nm, (B) 53.5 nm, (C) 107 nm and (D) all cores. The core diameters were analyzed assuming that they were spherical and the frequency and cumulative distributions are plotted. Particle size appears to be determined by the number of cores per particle rather than the size of those constituent cores.
3.4.3 - Surface Chemistry

The SPDP assay was used to spectrophotometrically determine the concentration of surface amines on the synthesized and modified particles. Consistent with expectations, the number of amines per particle increased with increasing diameter and therefore surface area. The values of the functional groups per particle for fully aminated particles is found in Table 3.1, summarizing the physical and chemical attributes of all investigated particles.

<table>
<thead>
<tr>
<th>Hydrodynamic diameter (nm)</th>
<th>Core diameter (nm)</th>
<th>Number of cores</th>
<th>$R_s$ (mM·s⁻¹)</th>
<th>$R_1$ (mM·s⁻¹)</th>
<th>$R_s/R_1$</th>
<th>NH₂ / particle</th>
<th>Fe (atom) / particle</th>
<th>Coating material</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>6.067</td>
<td>1.9</td>
<td>71.00</td>
<td>13.56</td>
<td>5.24</td>
<td>185</td>
<td>8924</td>
<td>Dextran</td>
</tr>
<tr>
<td>53</td>
<td>5.603</td>
<td>5.3</td>
<td>82.25</td>
<td>9.97</td>
<td>8.25</td>
<td>631</td>
<td>20065</td>
<td>Dextran</td>
</tr>
<tr>
<td>107</td>
<td>6.534</td>
<td>11.2</td>
<td>381.00</td>
<td>7.24</td>
<td>52.66</td>
<td>1024</td>
<td>66729</td>
<td>Dextran</td>
</tr>
<tr>
<td>207</td>
<td>175.4</td>
<td>1</td>
<td>176.58</td>
<td>0.51</td>
<td>344.48</td>
<td>6.09E+05</td>
<td>6.30E+07</td>
<td>Styrene Copolymer</td>
</tr>
<tr>
<td>289</td>
<td>289.6</td>
<td>1</td>
<td>115.20</td>
<td>0.34</td>
<td>337.43</td>
<td>2.20E+06</td>
<td>2.60E+08</td>
<td>Styrene Copolymer</td>
</tr>
<tr>
<td>1430</td>
<td>0</td>
<td>1</td>
<td>64.32</td>
<td>0.41</td>
<td>156.49</td>
<td>8.50E+08</td>
<td>1.30E+07</td>
<td>Silica</td>
</tr>
</tbody>
</table>

Table 3.1 - Physical and Magnetic Properties of SPIO

* $R_1$ values for 207, 289, and 1430 nm particles may be underestimated due to precipitation during measurements.
** Measurement of Fe (atoms) / particle for the commercial particles was made using the company provided relative iron mass per particle data, rather than the core size determination from TEM.

Blocking of the functional groups was accomplished through crude reaction with glycidol. Previous use of glycidol in the literature for use with NPs had centered on
attempts to use it to reduce the charge-dependent cytotoxicity of polymer NP such as dendrimers (26). This approach was adopted with the iron oxide NP and facile mixture with the agent consumed amines. Further, purification of the nanoparticles from excess spent or still-reactive glycidol was possible through the addition of isopropanol. Removal of the organic phase was complete after several washes.

Stability of the dextran shell was determined by an examination of the dextran that could be detected in solution (no longer complexed to SPIO) following residence in harsh conditions. The conditions used were meant to simulate accelerated lifetime of the particles in vivo, 80°C over roughly 100 hours. Two groups of samples from the same batch of SPIO, one having been cross-linked the other not, were run through a filter at various time points over the course of the heating. Using a colorimetric assay for the detection of polysaccharide, it was possible to determine the concentration of dextran that was being dissociated from the nanoparticle. The results, as shown in Figure 3.5, show that particles that had not been cross-linked gave off more dextran over time. Examination by eye also revealed that the non-cross-linked solution became cloudy after approximately 2 days of heating, while the cross-linked particle solution did not change. These results indicated that the cross-linking of the dextran shell significantly enhanced the stability of the particles.
The stability of the cross-linked (CLIO) and as-synthesized (MION, non-cross-linked) 33 nm SPIO is shown. Particles were filtered and the flow through volume was assessed for the presence of dextran. As-synthesized samples were found to continually release dextran, which is hypothesized to be from degenerating nanoparticles. Free dextran in the solution of cross-linked particles was detectable to some degree. It is possible that this is a background level of non-cross-linked dextran electrostatically-associated with a particle and/or free dextran not purified from the synthesis solution.
The magnetic characteristics of an MR CA are key to their application as in vitro and in vivo contrast agents. The R\textsubscript{1} and R\textsubscript{2} data (Figures 3.6A-B), indicate that there is a trend of increasing R\textsubscript{2} and decreasing R\textsubscript{1} with size up to the 107 nm particles. For particles of greater size, the single large core of the 207 nm and 289 nm particles does not result in proportionately higher R\textsubscript{2}. This likely reflects lower crystallinity of the larger single iron oxide cores in comparison to smaller crystals (27). Furthermore, according to the Solomon-Bloembergen theory, which relates the relaxation rate to particle properties, the total volume of the particle is not critical to the magnitude of R\textsubscript{2} as the susceptibility effect falls off from the surface with an exponential (r\textsuperscript{6}) dependence (28, 29).

It is hypothesized that as water has limited surface access, especially in the diffusive time span over which magnetic measurements (and images) are taken, such size is detrimental to dephasing of proximal protons. It should be noted that the R\textsubscript{1} values reported for particles greater than 200 nm are likely underestimates due to precipitation of the particles during T\textsubscript{1} measurements. For instance, determining T\textsubscript{1} relaxation times required more than 100 seconds per sample, which was ample time for the micrometer-sized particles to precipitate out of suspension.
Figure 3.6 - Relaxivity Measurements of SPIO.

SPIO of various size were diluted in PBS to iron concentrations between 1 and 200 µg Fe/mL for measurement at 1.41 T and 40˚ C. T₁ (A-B) and T₂ (C-D) values were then obtained. The inverse of the relaxation times, in seconds, was linearly fit against concentration to yield the particle R₁ and R₂. Non-linearity of 1430 nm particles in T₁ and T₂ measurements was a result of sedimentation during magnetic measurement.
3.5 - Conclusion

Iron oxide nanoparticles were prepared using a chemical coprecipitation synthesis. At the outset, reaction conditions were varied to determine a suitable protocol for the production of SPIO following good manufacturing processes. High quality particles, possessing attributes such as limited polydispersity, high transverse relaxivity, small size and long term colloidal stability were sought. In order to probe the effects, if any, of the modification of reaction conditions on the resultant particles detailed macroscopic and nanoscale characterization was performed. Measurements included the hydrodynamic and core size and mass, surface chemical functional density and magnetic relaxivity.

It was found that synthesis conditions could be reliably varied to produce particles over roughly an order of magnitude of size and multi-component core number. To complement our understanding of particle characteristics over a larger size domain, commercial particles were used to supplement the synthesized group. The number of surface amines and the iron per particle increased with greater particle diameter over the size scale from 33 nm to 1430 nm. Comparison of core and magnetic properties revealed that initial assumptions that greater size, and therefore greater iron content per particle, would result in larger NP cores and greater $T_2$ relaxivity were incorrect for the entire range of particle sizes. The number of cores, and thus the amount of iron per particle, was found to significantly influence the relaxivity of the laboratory-synthesized particles, but not the larger, commercially obtained, samples. For dextran-coated SPIO the number of cores was found to increase with hydrodynamic radii and ratio of iron to dextran, while constituent core size remained relatively constant. The transverse and longitudinal
relaxivities were greatest for the particle with the greatest number of cores and not the particle with the greatest iron content.
3.6 - References


CHAPTER 4. Size, Charge and Concentration Dependent Uptake of SPIO by Non-Phagocytic Cells

4.1 - Abstract

We have evaluated the cellular uptake of superparamagnetic iron oxide (SPIO) in non-phagocytic T cells over a continuum of particle sizes ranging from 33 nm to nearly 1.5 μm, with precisely controlled surface properties. Further, this has been accomplished without the need for potentially cytotoxic transfection agents. SPIO labeling of T cells was analyzed by flow cytometry and contrast enhancement was determined by relaxometry.

SPIO uptake was dose dependent and exhibited sigmoidal charge dependence, which was shown to saturate at different levels of cationic surface functionalization. Efficient labeling of cells was observed for particles up to 300 nm, however micron-sized particle uptake was limited. We have shown that an unconventional highly cationic particle configuration of aminated 107 nm diameter standard-SPIO (SSPIO) particles maximized magnetic resonance (MR) contrast of T cells. These SPIO surprisingly outperformed the widely utilized ultrasmall-SPIO (< 50 nm) configuration and demonstrated sufficient loading for subsequent in vivo visualization.
4.2 - Introduction

Continuing advancements in the fields of cell-based and cell-targeted therapy, such as engineered cytotoxic T cells or cell depleting antibodies, respectively, hold great promise for combating disease. Future development of these methods require greater knowledge than is currently available regarding the cellular distribution of the therapies and targets in vivo. This has led to the emergence of cellular imaging as a strategy to track the migration and biodistribution of target cells in living organisms. Pre-clinical studies have shown that cellular imaging can be used to evaluate stem cell distribution and homing in cell-based regenerative therapies (1, 2). Recently, cellular imaging has also allowed for improved assessment of functional efficacy and applicability of immunotherapeutic treatments in disease models for cancer (3-5) and AIDS (6). These studies have shown that the ability to determine the cellular effect of treatment, quantitatively and over long periods of time, using non-invasive approaches can have a significant impact on the development of therapeutic protocols.

In addition to evaluating cell-based therapies, cellular imaging has the potential to provide a great deal of insight into diverse physio- and pathological phenomena. Interesting applications include the observation of monocyte recruitment to atherosclerotic lesions for the mapping of disease development and therapeutic intervention (7), imaging embryonic stem cell movement during embryonic (8) and organ development (9) and the monitoring of metastatic cellular extravasation and tissue invasion (10, 11).
Tracking of labeled cells has been accomplished with a variety of imaging modalities including optical methods, positron emission tomography (PET), single photon emission computed tomography (SPECT), and MR (12-14). MR imaging presents a particularly promising approach because of its high spatial resolution in three dimensions and exquisite soft tissue contrast, which can be acquired concomitantly with the contrast-enhanced cellular distribution.

MR detection of cells in vivo is often accomplished following labeling with superparamagnetic iron oxide (SPIO) particles. SPIO are negative contrast agents that are typically composed of an iron oxide crystal core surrounded by a polymer or polysaccharide shell (15). A variety of manifestations of SPIO have been used to track cells, which can be broadly categorized as:

1) Ultrasmall-SPIO (USPIO) with an overall diameter of 30 - 50 nm (16)
2) Standard-SPIO (SSPIO) with a diameter of 50 - 250 nm
3) Micron-sized paramagnetic iron oxide (MPIO) having a diameter approaching or greater than 1 \( \mu \text{m} \) (17).

To date, USPIO have perhaps been the most widely utilized SPIO configuration for cell labeling. Although they provide less contrast enhancement per particle as compared to SSPIO and MPIO, large numbers of particles can be loaded into each cell, allowing for large magnetic susceptibilities to be generated (18, 19). Cationic surfaces have been shown to facilitate cellular internalization (20, 21), and thus USPIO are often modified with polycationic cell permeating peptides (CPP) such as HIV-Tat (22) or protamine (23). Other transfection techniques such as electroporation, sometimes in concert with CPPs, have also been used (24, 25).
An exciting new direction for cell tracking involves labeling cells with MPIO (26, 27). The large iron oxide cores present in these particles provide enough contrast for single cells to be imaged by MR. However, work with such large particles generally confines application of iron oxide-labeling to phenotypes such as macrophages (18), dendritic cells (28) or hepatocytes that actively internalize foreign material. MPIO uptake in non-phagocytic cells has been accomplished, however several non-trivial modifications to the particles were required. The additional conjugation steps and associated cost of using the antibody-mediated loading of MPIO is likely prohibitive to many for cell tracking purposes (29). As well, any such antibody targeting approach renders the contrast agents species specific and may induce adverse cellular events.

Recently, several studies have attempted to define an optimized particle configuration for iron oxide labeling of both phagocytic and non-phagocytic cell types. These attempts are part of a maturation of the nascent nanomedicine field to understand how NPs interact with biological systems. Most of this work has been directed at the role of NP chemical and physical attributes with respect to tumor targeting (30, 31). With respect to phagocytic monocyte loading, it was found that these cells are more effectively labeled with SSPIO (150 nm) compared with USPIO (30 nm) although MPIO was excluded from all of these studies (18, 32). Further, it was found that ionic carboxydextran-coated SSPIO (i.e. ferucarbotran) performed better than non-ionic dextran-coated SSPIO (i.e. ferumoxide) (18). It remains unclear how MPIO compares with these agents. It should be noted that the ability to conduct single cell detection using MR imaging systems has been achieved in phagocytic cells with both SPIO configurations (33, 34).
Chapter 4

The optimal SPIO configuration for labeling non-phagocytic cells has been much more elusive and findings have been contradictory. For example, in one study it was found that the delivery of carboxydextran SPIO and dextran-labeled SSPIO into non-phagocytic cancer cells and leukocytes (with the assistance of lipofection agents) was similar in terms of iron uptake (21). Both particles led to higher iron uptake than carboxydextran-coated USPIO. This indirectly suggests that larger particles with ionic coatings are superior to non-ionic USPIO. However, in a different study it was found that, in the presence of poly-L-lysine, ionic (aminated) USPIO exhibited significantly higher iron uptake in non-phagocytic cells compared with SSPIO. These data suggest that smaller ionic particles are internalized into non-phagocytic cells more efficiently (35). These contradictory findings likely stem from the variability and imprecise control over surface charge and the limited number of particle configurations examined, particularly with respect to diameter (ranging only from ~17 to 150 nm).

This chapter systematically evaluates the cellular uptake of SPIO in non-phagocytic T cells over a continuum of particle sizes ranging from 33 nm to nearly 1.5 μm and with precisely controlled surface properties, as detailed in the previous chapter. T cells were selected as a model non-phagocytic phenotype since visualization of their distribution is expected to be of importance for adoptive T cell therapy for cancer and T cell homing in autoimmune diseases. Extremely fine control was exerted on the surface properties of SPIO by direct chemical modification of particle surfaces rather than attempting to modulate the density of supplemental transfection agents. The main reason for this approach was to avoid the interfering and generally detrimental effects posed through use of charged peptide or cell membrane destabilization technologies.
Beyond the role of surface modification, concentration effects and incubation time were also tested in the interest of isolating the role particle size exerts on individual cell uptake and overall contrast enhancement. Our work shows that in a space between USPIO and MPIO exist configurations of relatively small particles (~100 nm) that efficiently label non-adherent, non-phagocytic T cells and generate higher relaxivity (per cell) relative to particles of other sizes.
4.3 - Materials and Methods

4.3.1 - Nano- and Microparticle SPIO

Rigorous characterization of the iron oxide contrast agents utilized in this in vitro study were detailed in Chapter 3. To recapitulate; nano- and microparticles of six different size configurations were analyzed for their hydrodynamic diameter, core size and composition, magnetic and surface properties. The particles are referred to by their overall (hydrodynamic) diameter throughout this chapter and include the laboratory synthesized 33.4 nm USPIO, 53.5 and 107 nm SSPIO and the commercially obtained 207 and 289 nm SSPIO and 1430 nm MPIO. All particles were functionalized with cationic amine groups. Further, we have demonstrated the ability to control the surface amine content of the laboratory-synthesized particles. Additional details regarding synthesis and modification protocols as well as characterization can be found in Chapter 3.

4.3.2 - Cell Culture and Labeling

Immortalized human T cells, Jurkat Clone E6-1 (ATCC), were maintained at 37°C in 5% CO₂ in RPMI 1640 (Mediatech, Manassas, VA) media supplemented with 10% FBS (Hyclone, Logan, UT) and penicillin/streptomycin (Mediatech). T cells were labeled with iron oxide particles (between 0 and 200 μg Fe/mL) by incubating the commercial and lab-made particles with 2 x 10⁶ cells in 400 μL of fully supplemented media for 1 or 4 hours, at 37°C in 5% CO₂. Cells were washed of non-internalized particles through two methods. Synthesized dextran-coated particles were washed from cells using centrifugation. Specifically, cells were pelleted at 0.5k rcf for 5 minutes and resuspended
in PBS. This was repeated three times. The dextran-coated particles are highly soluble in aqueous solvents and do not precipitate at these centrifugation speeds. Removal of non-internalized commercial particles was accomplished through a density gradient. The cells and particles were diluted to 1 mL with PBS and overlayed on 4 mL of room temperature Ficoll-Paque PLUS (GE Healthcare). The sample was centrifuged at 0.4k rcf for 40 minutes. Cells loaded with particles were retrieved from the interface layer.

To determine if particles were internalized or merely adsorbed on the cell exterior, surface receptor cleavage enzyme trypsin was used. Following particle incubation, as described above, cells were exposed to 0.025% trypsin-EDTA (Invitrogen) for 5 minutes. Purification of non-internalized particles was carried out as detailed. No statistical difference was seen in either flow cytometry or relaxometry between groups washed with or without enzyme.

4.3.3 - Assessment of Labeling

Immediately after non-internalized iron oxide particles were removed from T cell samples, flow cytometry was performed on a Guava Easycyte (Guava Technologies, Hayward, CA). For labeling and viability experiments, forward and side scattering were used to identify the entire population of cells. Data analysis of flow cytometry data was accomplished with FlowJo (TreeStar, Ashland, OR). Viability of T cells was determined using the LIVE/DEAD cytotoxicity kit for mammalian cells (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Specifically the proportion of dead cells was determined using the membrane-impermeant ethidium homodimer-1. This dye labels
nucleic acids of membrane-compromised cells with red fluorescence. In order to evaluate the decrease in T$_2$ relaxation time of iron oxide internalized in T cells, purified cells were lysed for 30 minutes in 0.1% SDS in PBS at 37°C. Samples were diluted to 0.5x10$^6$ cells/mL in 300 uL and T$_2$ relaxation times were measured using a benchtop Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz at 40°C; Bruker Optics Ltd, Billerica, MA). All flow and magnetic resonance measurements were made in triplicate on at least two separate occasions.
4.4 – Results

4.4.1 – Cell Loading

The extent to which T cells internalize iron oxide particles is dependent upon more than mere particle size. Various other particle characteristics and cell loading conditions, including surface charge, particle concentration, and incubation time play a role. Thus, before it could be determined which particle size or configuration led to the highest relaxivity per cell, it was first necessary to identify conditions whereby cell loading was independent of these other parameters.

In the current study, all SPIO samples were fluorescently labeled with an equivalent amount of FITC/iron. The use of fluorescently labeled iron oxide particles combined with flow cytometry provided a facile method by which particle uptake could be systematically assessed in a high-throughput manner. Following the exploration of concentration, time and charge dependent uptake parameters, the contrast enhancement as analyzed by magnetic relaxivity was determined.

4.4.2 - Concentration Dependence

In order to confirm that iron oxide particles were present in sufficient quantity for maximum cellular uptake, T cells were incubated with increasing iron concentrations until a saturating level was reached. As shown in Figure 4.1, dextran-coated particles were efficiently internalized, all reaching a plateau at iron concentrations below 50 μg Fe/mL. Greater than 100 μg Fe/mL was required to saturate the loading of the 207 nm, 289 nm and 1430 nm particles. The necessity for these higher iron concentrations may be
attributed to the fact that the number of particles per unit of iron is far less than the smaller agents. Further, there is likely less contact between the larger particles and suspended cells because of their continual sedimentation. This was perhaps most evident with MPIO, where cell labeling was poor across all particle concentrations. Even at 1000 μg Fe/mL (data not shown) labeling with MPIO did not reach the loading levels achieved by the dextran-coated USPIO and SPIO.
Figure 4.1 - Dependence of SPIO Loading on Particle Concentration.

Fluorescently labeled SPIO of various size and across a range of concentrations were incubated with $2 \times 10^6$ T cells/mL at 37°C for 4 hours (excluding the 107 nm particle as indicated). SPIO uptake was then measured by flow cytometry. Each experiment was conducted in triplicate on at least two separate occasions and each data point represents the average value for the mean fluorescent intensity (MFI). Note the difference in x- and y-axes for (A) and (B). All particles were maximally aminated (for values see Table 3.1).
4.4.3 - Role of Particle Charge

Surface charge is important for intracellular delivery of exogenous material. This principle has been described for a variety of nanoparticles (examples include gold (36), polymer (37, 38) and silica (39)) and biologicals (for example delivery of DNA with cationic proteins, lipids and polymers (40)) contexts. It stems from the realization that charged peptides enabled non-receptor mediated viral uptake (41). The aminated surfaces of the particles used in this study provide an inherent surface charge, facilitating cellular interaction. However, in order to study the role this property has in the intracellular delivery of iron oxide contrast agent, it was necessary to manipulate the magnitude of the surface charge. To do so we have applied glycidol, a hydroxyl terminating epoxide, to generate subsets of particles with a gradient of surface amines.

Glycidol has been used previously in dendrimer chemistry to reduce the chemotoxicity of highly-positively charged dendrimers (42). The tight control of surface properties produced by consuming amines with glycidol allows for isolated examination and evaluation of the role of surface charge on SPIO.

The summary of particle uptake on a per cell basis is shown in Figure 4.2A-C. Each data point represents the normalized mean fluorescence intensity (MFI) of T cells that were incubated with iron oxide particles at a saturating concentration (previously determined) for 4 hours. Under these incubation conditions, it was found that particles in their natural (fully aminated) state are maximally internalized. Any further increase in the positive surface charge will not further augment SPIO loading. In other words, the efficiency of cell labeling has become independent of surface charge. In all cases, uptake
and internalization of the particles was rapid. Representative uptake of the 107 nm particles as a function of time is shown in Figure 4.2D.
T cell uptake of fluorescently labeled SPIO as a function of surface charge was examined by modulating the number of amines per particle for the (A) 33.4 nm, (B) 53.5 nm and (C) 107 nm particles. A gradient in the degree of functionalization was produced by glycidol blocking of amines. SPIO were incubated with T cells at saturating concentrations, 50 μg Fe/mL, under identical conditions. Flow cytometry was then performed to assess the relative uptake of each SPIO. Each data point represents the mean fluorescent intensity (MFI). The loading of SPIO was rapid; (D) shows the representative uptake of fully-aminated 107 nm particles as a function of time.
4.4.4 - Cell Viability

The impact and potential cytotoxicity of each iron oxide particle on T cells was measured using a fluorescent cell viability marker. Negligible to low levels of cell death were observed (Figure 4.3) for all particles at diminished and saturating concentrations of iron oxide (10 and 50 µg Fe/mL, respectively). The exception was for the 107 nm SPIO, which exhibited some adverse cell influence even at 10 µg Fe/mL. This effect was exacerbated at increased concentrations. When the amines on the 107 nm particle were completely blocked, cell death was reduced to negligible levels; however, internalization was also reduced to negligible levels (Figure 4.2C).

T cell death is likely attributable to the high positive surface charge possessed by the SPIO. Similar results have been seen with amine-terminated poly(amidoamine) dendrimers (43). The extremely high driving force for cell internalization imparted by positive SPIO surface charge can lead to cell death. We speculate this occurs through non-reversible membrane degeneration resulting in either fatal cytoplasm leakage or active cell death in response to highly charged foreign bodies.

In order to minimize the toxicity of the 107 nm particles, the incubation time with T cells was decreased to 1 hour. As shown in Figure 4.2D, particle uptake is still saturated within this time frame; therefore exposing T cells to excess SPIO for longer periods of time was deemed unnecessary. No toxicity was observed with the 107 nm particles after just 1 hour of incubation.
Figure 4.3 - Viability of T Cells Incubated With SPIO.

SPIO were incubated with T cells at various iron concentrations: 10 μg/mL [black], 50 μg Fe/mL [white], and 100 μg Fe/mL [grey]. After 4 hours (unless otherwise noted), viability was measured and normalized to cells grown in the absence of any particles (blank). All SPIO exhibited negligible impact on cell survival after 4 hours, excluding the 107 nm diameter particles. Reducing incubation time of these particles to 1 hour eliminated adverse effects at both low and saturating concentrations.
4.4.5 - Contrast Enhancement

Flow cytometry was utilized to determine the saturating conditions for each SPIO; however, these single cell measurements were conducted with some variation between the number of fluorescent labels per particle making it difficult to accurately quantify the number of particles per cell. Also, after labeling cells with superparamagnetic tracking agents the critical assessment of ability to track cells is their relaxivity. Therefore, a benchtop NMR spectrometer, near to the clinical field strength of 1.5 T, was utilized for evaluating in vitro loading. As shown in Figure 4.4, T cells loaded with particles showed a dose-dependent, negative contrast enhancement.

As befits their widespread application in the literature, the USPIO proved effective at lowering the spin-spin relaxation time ($T_2$). Despite delivering only a small payload of iron per particle, the large numbers of 33.4 nm and 53.5 nm particles that accumulate in the cells allows for a strong aggregate effect, producing an average $T_2$ signal of 126.05 msec and 51.5 msec under saturating conditions, respectively. These reduced signal values correlate to an 8.04 and 19.68 times reduction in signal from T cells without any contrast agent ($T_2 = 1013$ msec).

Performance of particles greater than 200 nm was ranked inversely with diameter. Greater concentrations of large particles continued to reduce the $T_2$ signal; however, when the iron concentration was increased above 500 $\mu$g Fe/mL the methods used to distinctly separate loaded-cells from free particles became less reliable. It should be noted that this drawback does not exist for the flow cytometry measurements, as the particles themselves could be excluded from the cells based on forward and side scatter. At 150 $\mu$g
Fe/mL, the spin-spin relaxation signal from the 207 nm, 289 nm and 1430 nm particles were 149.75 msec, 224.3 msec and 398 msec, respectively. These finding suggest that despite their high R₂ values and large iron content, particles greater than 200 nm seem to have limited applicability in labeling non-phagocytic cells.

The highly-aminated SPIO with a diameter of 107 nm produced the greatest contrast enhancement. These particles combined the high degree of internalization of the USPIO with the superior relaxivity of larger particles. At the 1 hour loading time, to avoid any longer term cytotoxic events, these SSPIO were able to reduce signal by approximately two orders of magnitude, providing T₂ signal of only 12.25 msec, or a 82.74 times reduction in signal from control. This reduction in signal was approximately 5 and 10 times greater than that produced by the 53.5 nm and 33.4 nm SPIO (for the same concentration).
Figure 4.4 - Contrast Enhancement.

T₂ Relaxation times of T cells labeled with SPIO. T cells were labeled with SPIO of various size and across a range of concentrations. The T₂ relaxivity of 0.5x10⁶ SPIO-loaded T cells/mL in 300 μL was measured on a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz). The signal decrease observed following internalization of SPIO is dose dependent and saturation correlates well with values determined by flow cytometry. The 107 nm SSPIO produced maximum signal decrease.
4.5 - Conclusions

In this work, efficient iron oxide labeling, without the use of cell penetrating peptides or transfection agents, was accomplished in a clinically relevant non-phagocytic cellular system. The level of SPIO loading in T cells was determined by flow cytometry and verified through evaluation of MR contrast enhancement. Using conditions under which cell loading was independent of particle concentration, chemical surface modification, and incubation time, particle size was isolated as an attribute to affect nano- and microparticle loading.

Large particles, over 200 nm in diameter, possess much greater amounts of iron per particle, and thus theoretically require few or single particles per cell in order to be detected. However, they suffered from gravitational sedimentation, decreased efficiency of cell labeling, and in some cases free particles were incompletely removed from cells. This may not be a problem with adherent and/or phagocytic cell systems, but significantly hampered their use as magnetic probes for non-phagocytic suspended cells.

The vastly greater number of USPIO that accumulate within the cells made up for their weaker $R_2$ values. While a general trend correlating increased or decreased particle size with labeling was not observed, it was clear that the 107 nm SPIO manifestation led to the largest $T_2$ signal decrease. The observation that SSPIO provide maximal contrast amongst the SPIO configurations tested here is surprising given the ubiquity of USPIO and current vogue of MPIO in the literature. These results may have a significant impact on the SPIO configuration used to label and subsequently track non-phagocytic cells in vivo.
4.6 - References


8. Hadjantonakis AK, Papaioannou VE. Dynamic in vivo imaging and cell tracking using a histone fluorescent protein fusion in mice. Bmc Biotechnology 2004; 4:-.


CHAPTER 5. B Cell Tracking in SLE Autoimmune Therapy Model

5.1 - Overview

Antibody mediated B cell depletion has been applied successfully to treat non-Hodgkin's lymphoma (1). This strategy has been extended in trials to treat systemic autoimmune diseases which also have B cell related pathologies. When applied to systemic lupus erythematosus (SLE), a disease in which loss of B cell tolerance plays a central role (2-5), B cell depleting antibody therapy has not yet demonstrated clinical efficacy (6-8). Appreciation of the biological response to treatment would be beneficial for greater understanding of underlying disease mechanisms and insight towards development of effective therapies (9). Cellular and molecular imaging approaches to track the in vivo distribution of B cells would allow for the repeated, non-invasive, and quantitative determination of the extent of B cell depletion therapy effect and duration in an intact animal.

We have made use of an ex vivo labeling approach to enable small animal magnetic resonance (MR) and fluorescence imaging to accurately determine B cell distribution in vivo. Superparamagnetic iron oxide (SPIO) and CellVue 815 near infrared (NIR) dye were loaded into B cells and subsequently administered to mice. Distribution of cells over a time course of 15 days, with and without the introduction of B cell
depleting anti-CD79, was ascertained. The presence of the cells in the spleen was observed longitudinally, and quantitatively analyzed between treated and non-treated groups. Ex vivo fluorescence of organs of interest as well as immunohistology of the spleen was performed in order to describe the fate of the B cells in this system. It was seen that the cells loaded with SPIO and CellVue 815 were less responsive to immunotherapy, as compared to cells labeled with CellVue 815 alone. Ultimately, it was determined that SPIO were interfering with the depleting function of the antibody, despite their apparent lack of effect in vitro on cell viability and function.

This chapter begins with an introduction to the motivation behind our work on tracking lymphocytes and the clinical issues associated with the application of B cell depleting antibody to SLE. Methods of lymphocyte tracking are detailed, with an emphasis on optical and MR strategies. The development of our approach, the applied scheme and techniques are comprehensively discussed. Following this introduction, the results and implications of this work are discussed.
5.2 - Background; Depletion Therapy

5.2.1 - Anti-CD20 and Non-Hodgkin's Lymphoma

Rituximab is a chimeric anti-CD20 monoclonal antibody used to effectively treat B cell non-Hodgkin’s lymphoma (1). The antibody consists of the human IgG1 and kappa constant regions along with mouse variable regions from a hybridoma directed at human CD20. The mechanism of action of the antibody for therapy is to target and deplete the CD20+ sub-population of B cells in the patient (10, 11). The exact biological role of CD20 has not been defined (12, 13), however it is suspected to play a role in signaling (14). Further, the means by which the antibody binding of this membrane-surface protein is able to deplete the cells in vivo has not been fully elucidated, although the consensus view is that cell death occurs by more than one mechanism (12, 15).

The primary methods through which B cells are depleted by anti-CD20 include apoptosis, complement dependent cytotoxicity (CDC) and antibody dependent cell mediated cytotoxicity (ADCC). Following administration of rituximab, the in vivo activation of caspase-3 and -9 have been noted in the blood leukemia cells of patients (16). It has been shown that this effect is dose and time dependent (17). CDC activity has been observed as an antibody mediated means of cell disposal. CDC activity, including complement activation (18), has been correlated to tumor cell death (19) and translocation of CD20 to lipid rafts (20). Finally, ADCC events mediated by FcγR have been shown to be important for B cell depletion. Rituximab has been shown to bind activation receptors for effector cell mediated cytotoxicity (21). Other means by which the antibody could deplete the cells, such as antiproliferative effects, have also been
Chapter 5

suggested (22). The success of rituximab for treatment of B cell lymphoma has encouraged its application in other B cell pathogenic diseases.

5.2.2 – Rituximab and SLE

B cells are a key component of the adaptive immune system and are responsible for the maintenance of cellular and humoral protective memory. Autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus (SLE) and multiple sclerosis, are associated with the presence of B cell hyperactivity and/or dysfunction (23). Their role is central; it has been shown that autoimmune prone mice that lack B cells do not develop characteristic autoimmune features such as kidney destruction, vasculitis or autoantibodies (24).

SLE is a systemic autoimmune disorder characterized by autoantibody production, not restricted to a single organ system, and inflammation of the joints, skin, kidneys, heart, lungs, blood vessels and central nervous system. B cells are critical actors in the immunopathogenesis of SLE even beyond the production of autoantibody (25, 26). Pathogenesis is believed to be mediated through the B cells’ multiple roles as an antigen presenting cell, involvement in T cell activation and production of cytokines (27, 28).

Rituximab is able to reduce B cells from the peripheral blood nearly completely on the order of months and has been tested as a treatment for SLE (29). Early results were promising (30-32), however clinical trials of the antibody have been disappointing (6-8). For example, a collaborative Genetech and Biogen Idec multicenter Phase II/III trial in 2008 did not meet any of six endpoint goals demonstrating Rituxan (rituximab; Roche Genentech, Inc, South San Francisco, CA and IDEC Pharmaceuticals, San Diego, CA)
efficacy over placebo as measured by the British Isles Lupus Assessment Group (BILAG) index. This metric is a validated clinical measure of lupus disease activity.

### 5.2.3 – Beyond Rituximab for SLE Immunotherapy

Additional approaches to B cell depletion as a treatment for SLE are in development. These include other B cell cytotoxic antibodies that target process critical to B cell function. For example, belimumab is a human monoclonal antibody that binds to and inhibits the biologic activity of BLyS, which is essential for B cell survival (33, 34). The fully human nature of the antibody reduces some safety concerns associated with rituximab over long treatment regimes (81), which may lead to production of human anti-chimeric antibody (35). Pre- and clinical results to date show that belimumab and epratuzumab (another humanized monoclonal antibody; targeting CD-22 (36)) can reduce the levels of circulating CD20⁺ B cells (34). However, the circulating B cell depletion is apparently less effective than rituximab (37). Another depleting antibody, anti-CD79, targets the signaling complex of the B cell receptor (38). In mouse models of autoimmune disease, it has been shown that B cell depletion was extensive and contributed to enhanced survival (39).

Counts of peripheral blood lymphocytes are commonly used to assess anti-CD20 therapeutic effect. This method provides a quantitative cellular measurement of global circulating B cells. However, it is insufficient in determining true therapeutic impact as depletion from lymphoid tissues has been shown to be highly variable (40, 41). Failure to deplete in these tissues may well lead to relapse of, or unabated disease. It has become
apparent that the overall biological impact of the therapy, during the course of treatment, must be better understood (9).

In animal models, it is possible to harvest spleens and lymph nodes for lymphocyte subset analysis. However, such an approach is inherently limiting as it presents an ex vivo, terminal view of these organs. We hypothesize that methods to measure B cell depletion repeatedly in the intact patient or animal would be of great pre- and clinical interest. Such techniques would allow for the determination of effectiveness of a treatment at the cellular level and correlation to clinical benefit without sacrifice. The next section will provide a detailed account of attempts to track lymphocytes, and B cells in particular, across a range of imaging modalities.
5.3 - Background; Lymphocyte Tracking

A variety of methods have been used to study the in vivo distribution of lymphocytes in animal models and human patients. These include radioactive tracers, biological reporters, fluorescent moieties and magnetic resonance (MR) contrast agents. This section will provide a description of these techniques.

5.3.1 - Radioactive Lymphocyte Tracking

A convenient method for the general overview of the distribution of injected lymphocytes is through the use of radiotracers. Indium-111 labeled lymphocytes were used to image biodistribution of radiation treated cells over 20 years ago (42). The most commonly applied clinical radiolabel, $[^{18}F]$fluoro-deoxy-glucose (FDG), has been used in a limited number of studies to track lymphocytes, following incorporation in vitro (43). Major drawbacks associated with this approach are the low incorporation of radiotracer and, as with all radioactive based imaging, the loss of positron-emitting substance (FDG) over time (as much as 20% over 1 hour) (44). This limits the duration over which imaging of distribution can be performed. Further, there are radioactive health related concerns for both the animals and scientists involved in such studies.

5.3.2 - Biological Reporters

Several molecular biology constructs have been employed to enable in vivo visualization of lymphocytes. These include fluorescent and bioluminescent proteins which have been used to reveal information about T and B cell development, interaction
and distribution (45, 46). Bioluminescent imaging (BLI) is a particularly attractive platform for such molecular imaging applications due to its simplicity, sensitivity and the ability to obtain temporal information (47). Relevant to the greater understanding of the use of anti-CD20 antibodies, the luminescent approach has recently been used to quantify the dose dependent effect of rituximab on luciferase-expressing lymphomas (48). However, use of such constructs is hampered by the need for extensive animal model development, depth penetration issues and general limitation to rodent models.

5.3.3 - Optical Methods

Optical tracking methods for the determination of lymphocyte distribution have been practiced for over two decades (49). Dyes used for B and T cell tracking can be classified into general categories; DNA-binding (50), cytoplasmic (51, 52), covalently coupled (53) and membrane-inserting dyes (54, 55). A table, listing commonly used lymphocyte tracking dyes is provided in Table 5.1. Initially, ex vivo dye-labeled cell populations' distribution was resolved using microscopic techniques at specific time points of interest. This approach is inherently limiting as it only allows for proliferation and migration to be studied in a terminally invasive fashion (52, 56). In a related approach, flow cytometry can be utilized for the detection of fluorescently labeled cells from the blood (or tissues processed into single cell suspensions). This technique provides an optical means to quantitatively determine, with high sensitivity, lymphocyte proliferation characteristics (57).
<table>
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<td>786</td>
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Table 5.1 - Fluorescent Dyes Used for Lymphocyte Tracking

Common names of dyes used for T and B cell studies in vivo and in vitro. Expanded by Thorek, from (49).

A powerful advantage of optical methodologies is that multiplexing is possible. This is especially advantageous given the large library of dyes, as seen in Table 5.1. The labeling of cells with distinct dyes enables subsequent visualization of distinct populations at the same time (58). In one such approach reported by Roy, et al., two near infrared dyes were used to concomitantly image distinct lymphocyte populations in mouse lymphoid and brain tissues (54). An issue that accompanies all dyes is that their persistent staining is difficult to accomplish. This can be an advantage for proliferation...
studies, where the loss of signal (read from a daughter cell) can be used to estimate the number of divisions from the labeled cell (59, 60). More often though, the loss of signal hinders long experimental duration. This also complicates interpretation of results as it is difficult to discern if reduced signal is from loss of cells or loss of dye. Furthermore, many of the dyes do not maintain their brightness over longer study lengths and may also have toxicity issues (57, 61).

5.3.4 - MR Imaging of Lymphocytes

Radioactive methods suffer from poor spatial resolution and the requirement of additional safety precautions. Optical imaging offers the advantage of high resolution, but suffers from tissue penetration depth issues. An alternative is magnetic resonance (MR); an attractive modality with translational capabilities for the long term tracking of cell populations. High-resolution images over long periods of time, without harm to the subject, can be performed. To track cells of interest, it is required that contrast agent used be labeled to the lymphocytes. This is more difficult than the labeling of innate immune cells, such as macrophage which, as part of their function in the immune system actively uptake foreign particles. Several strategies have been developed in order to track populations of lymphocytes in vivo by MR which can be categorized as either 1. In vivo targeting or 2. Ex vivo loading.

In vivo imaging using to T and B cell targeted-SPIO has previously been accomplished. Cell selective labeling in vivo with SPIO-antibody conjugates targeting cells directed by antibody (anti-CD-8, anti-CD-4 and anti-Mac1) enabled specific
labeling of lymphocytes in a CNS inflammation model (62). Anti-CD20 has also been coupled to USPIO for in vivo detection of CD20 expressing tumors (63).

In an effort to avoid conjugation of targeting ligands to nanoparticles, charge based strategies have been pursued to load contrast. Functionalization of SPIO, covalently or through electrostatic attraction, with cationic materials has been shown to enable lymphocyte uptake. T cells have been labeled with SPIO particles using poly-L-lysine (64) as well as protamine and the HIV-tat peptide (65). The cationic material mediated loading of particles takes place in vitro and cells are subsequently injected into another (or back into the cell donating) mouse for subsequent imaging and analysis.

Labeling of lymphocytes without the use of cationic mediators has also been demonstrated in several ex vivo loading studies. Conjugation of SPIO to a T cell receptor specific ligand enabled high levels of uptake of these probes through endocytosis into T cells (CD8+). In vivo delivery of these cells demonstrated longitudinal imaging of migration to the pancreas in a mouse diabetes model (66). However, the use of cell specific ligands is restrictive as it involves additional chemical conjugation and purification steps that result in drastically lower yields.

Visualization of T cells labeled with ultrasmall SPIO (USPIO) has been achieved ex vivo, without resorting to cationic or targeted labeling means (67). These citrate coated particles were loaded into tumor directed T cells ex vivo and subsequently implanted to allow for the high spatial and temporal tracking to the target and non-specifically to the spleen. Positive contrast detection of lymphocytes, likewise targeted to tumors, has been demonstrated using manganese salts (the most common T1-w contrast agent after gadolinium) (68).
It was shown in Chapter 4 that contrast agent accumulation in non-phagocytic lymphocytes (cultured human T cells) is possible for a variety of particle formulations. It was thus possible to determine the loading factors and SPIO characteristics that lead to optimal cell labeling for MR contrast enhancement (69). We hypothesize that this in vitro approach can be extended for the sensitive detection and tracking of lymphocytes in vivo. The robust contrast agent loading of lymphocytes possible using the developed ex vivo protocol will enable longitudinal, non-invasive in vivo tracking.
5.4 - Study Design

Our motivation was to determine whether SPIO enhanced MR could be used to monitor the efficacy of B cell antibody depletion therapy in a mouse model. We sought to visualize - with high spatial resolution over long times - the migration of B cells using magnetic resonance (MR) imaging and optical approaches. Four groups of mice were studied, as indicated in Table 5.2. Each possessed different conditions, with respect to the combination of contrast employed and/or treatment administered.

<table>
<thead>
<tr>
<th>Group</th>
<th>GFP B cells</th>
<th>NIR dye (contrast)</th>
<th>SPIO (contrast)</th>
<th>Anti-CD79 (treatment)</th>
<th>PBS (treatment)</th>
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Table 5.2 - Study Groups.

This framework enabled us to determine if any interfering effects of the SPIO existed regarding initial distribution or response to treatment. All contrast agents were loaded into purified GFP B cells in vitro, as illustrated in Figure 5.1A. Validation of the non-invasive imaging data was made possible through the histological co-localization of implanted cells, surface NIR dye and internalized SPIO. The use of GFP cells allowed for the immunohistological staining of B cells in the spleen to be specific for the introduced population.

Specifically, C57BL/6 mice were imaged over the course of 15 days (Figure 5.1B-C). The contrast-loaded cells were introduced following an initial precontrast
imaging session. One day following the B cell injection, a treatment of anti-CD79 or PBS was administered and the effect that each treatment had on the distribution and intensity of MR and fluorescent signal was quantitatively measured.
Figure 5.1 - Schematic.

(A) SPIO particles functionalized with amines and subsequently Alexa 680, along with membrane intercalating near infrared (NIR) dye CellVue NIR815, were incubated with murine transgenic-GFP B cells. (B) The contrast loaded cells, tail vein injected at $t=0$ into C57BL/6 mice, enabled longitudinal imaging by MR and optical techniques. GFP markers in the implanted B cells allowed for histological correlation of signal and cells ex vivo. (C) Imaging (in vivo and ex vivo) time points are indicated. Treatment of either PBS or Anti-CD79 (as indicated) was administered following the $t=1$ imaging session.
5.5 - Materials and Methods

5.5.1 - Nanoparticle Synthesis and Modification

Nanoparticles that enabled high levels of MR contrast agent loading into non-phagocytic lymphocytes had been previously prepared (Chapters 3, 4). Briefly, 53.5 nm diameter iron oxide nanoparticles coated with dextran were synthesized using the coprecipitation technique. All materials were purchased from Fisher Scientific (Hampton, NH) unless otherwise noted. To 50 mL of dH₂O, 25 g of dextran T10 (GE Healthcare; Piscataway, NJ) was dissolved and heated to 80˚ C for 1 hour. After full polysaccharide dissolution, mixing overnight and cooling to 4˚ C on ice it was degassed with N₂. Iron chloride salts, FeCl₂ (1.5 g) and FeCl₃ (4 g), were each rapidly dissolved in 12.5 mL of degassed dH₂O and kept on ice for approximately 10 minutes and added to the dextran solution. After 30 min of mixing, 15 mL of ammonium hydroxide was added. The resulting black viscous solution was then heated to 90˚ C for 1 hour then cooled overnight, followed by ultracentrifugation at 20k RCF for 30 minutes. The supernatant was continually diafiltered using a 100 kDa MWCO cartridge (GE Healthcare) on a peristaltic pump (E323, Watson Marlowe Bredel; Wilmington, MA). The particles were exchanged into 0.02 M citrate, 0.15 M sodium chloride buffer until all unreacted products had been removed.

The dextran-coated SPIO were cross-linked in base (25% v/v 10 M NaOH) using epichlorhydrin (33%) (65). This solution was mixed for 24 hours. Prior to amine functionalization of the cross-linked dextran shell, a brief 10 minute dialysis into 0.02 M citrate buffer was performed (8-14 kDa MWCO, 50 mm flat width; regenerated
Following the dialysis, additional ammonium hydroxide was added (25%) to the activated nanoparticles and mixed for 24 hours. The particles were then exhaustively purified via diafiltration, against 0.02 M citrate. The resulting particles were amine functionalized cross-linked iron oxide, SPIO.

Alexa Fluor 680 (Invitrogen; Carlsbad, CA) was dissolved in dimethylformamide and added to SPIO suspended in pH 9 sodium bicarbonate buffer. The final volume of the dye was 10% at a 10:1 molar labeling ratio of dye:SPIO. The solution was mixed overnight and then purified twice through a PD10 gel filtration column (GE Healthcare) equilibrated with phosphate buffered saline (PBS). Conjugation was verified by absorbance using a Cary-100 UV-Vis spectrophotometer (Varian; Palo Alto, CA). For preliminary experiments, fluorescein isothiocyanate (FITC, Pierce Biotechnology; Rockford, IL) was used in place of Alexa Fluor 680 under identical reaction and purification conditions.

The cross-linked cationic SPIO functionalized with fluorescent dye will be referred to as SPIO. This convention is adopted for simplicity and the fact that no other nanoparticles were utilized in this study.

5.5.2 - Cell Acquisition

Splenocytes were obtained from C57BL/6-Tg(UBC-GFP)30Scha/J (GFP) and C57BL/6 mice. Animals were culled and their spleen's removed. The spleen was macerated through a 70 μm strainer placed in a shallow dish. A single cell suspension was made by running the cells, in Hanks Buffered Saline Solution (HBSS), repeatedly through a 70 μm screen. The cells were pelleted by centrifugation at 4°C, 0.6k rcf for 5
minutes and resuspended in ACK lysis buffer (Invitrogen), placed on ice for 5 minutes and centrifuged again.

When required, B cells were purified from splenocytes, derived from the GFP mice, using a negative selection (70). Harvested splenocytes were centrifuged and resuspended in PBS + 0.5% BSA + 2mM EDTA (90 μL for every 10⁷ cells). The cells were then mixed and incubated with CD43 selection microbeads beads (30 minutes) and isolated using an autoMACS (Miltenyi Biotec; Hamburg, Germany). B cells were those that failed to bind (i.e. CD43 negative).

5.5.3 - Cell Labeling

B cells were incubated with SPIO (50 μg Fe/mL) in fully supplemented RPMI 1640 (ATCC; Manassas, VA) for 2 hours at 37° C, 5% CO₂. The cells were washed of non-internalized SPIO and prepared for CellVue NIR815 dye (NIR, Molecular Targeting Technologies, Inc; Malvern, PA) labeling by precipitation at 0.5k RCF and resuspension in protein free media, in triplicate. NIR membrane labeling was conducted following manufacturers instructions; NIR was diluted from stock by 1/50 to 16 μM in Diluent C, an iso-osmotic solution with no physiological salts or organic solvents (provided by manufacturer). The cells were added to this solution rapidly, mixed thoroughly and incubated covered from light for 4 minutes. Cells were washed from free dye by repeated centrifugation and resuspension into serum-supplemented media and subsequently PBS. Fresh 15 mL conical tubes were used for each wash to avoid dye binding to the vessel's hydrophobic walls. For preliminary experiments, the dye PKH26 (Sigma; St. Louis, MO) was used under identical loading conditions. PKH26 is a similar lipophilic dye, from
which NIR was derived.

Success of loading cells with SPIO and dye was verified by fluorescence microscopy. An Olympus IX 81 motorized inverted fluorescence microscope was used, equipped with an Ixon (Andor Technology PLC; Belfast, Northern Ireland) monochrome digital camera, an X-Cite 120 excitation source (EXFO; Quebec, QC) and Sutter excitation and emission filter wheels.

5.5.4 - Cell Activation

Activation of primary C57BL/6 B cells or splenocytes by incubation with SPIO was assessed by flow cytometry. In initial experiments to determine whether the SPIO loading would lead to activation of the B cells, the cells were cultured with SPIO (as above), washed and maintained in culture for 24 hours. Cells cultured with the SPIO for a total of 24 hours and cells cultured with increasing doses of PMA (phorbol ester, phorbol 12-myristate 13-acetate; 0.1-10 μg/mL) served as positive control. Cells incubated without additives represented the negative control. Activation was measured by changes in surface marker expression. Specifically, fluorescently labeled antibodies against CD40 (PE), CD86 (PE), CD80 (FITC) and MHCII (FITC) were used to label cells after triplicate purification from culture media to 0.1% BSA in PBS, on ice for 15 minutes (1/200).

The ability for B cell activation after SPIO loading was determined by loading splenocytes with SPIO, washing of excess nanoparticles and then incubating with LPS (E. Coli 055:b5; 0.01 – 10 μg/mL) in fully supplemented media. B cells were identified from the general splenocyte population by anti-CD19 (APC), and surface marker
expression was assessed as above. Flow cytometry was performed using a FACSCalibur (BD Biosciences; Franklin Lakes, NJ).

5.5.5 - Animal Procedures

All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (National Research Council, 1996) and approved by both the University of Pennsylvania's Institutional Animal Care and Use Committee and the Small Animal Imaging Facility (SAIF) Animal Oversight Subcommittee. All in vivo fluorescence and magnetic resonance imaging procedures were conducted at their respective sub-cores at the SAIF at the Department of Radiology in the School of Medicine.

Four groups of 8 adult (8 week old) male C57BL/6 mice (all mice obtained from Jackson Laboratories) were placed on AIN-76A low-autofluorescence rodent diet, ad libidum (Research Diets, Inc.). Six days after food change, animals were tail vein injected with 20M primary B cells derived from GFP-transgenic mice loaded with either SPIO and NIR or just NIR.

Three subjects per group were imaged by MR and whole-body fluorescent imaging at each of the following time points; prior to administration of B cells and , 1 hour, 1 d, 3d, 5d, 7d, 10d and 15 d post-injection. B cell depletion was conducted using an intraperitoneal injection of 250 μg of Anti-CD79β (HM79-16, Armenian hamster IgG (39, 71)) immediately following the conclusion of the day 1 imaging session. A peripheral blood lymphocyte measurement and necroscopy, for ex vivo fluorescent organ imaging, were performed on one mouse per time point, per group.
5.5.6 - In Vivo Fluorescence Imaging

Mice were induced with inhalation anesthesia, using a 4% mixture of Isoflurane in oxygen. Mice were maintained at a 2% mixture of the gas and shaved on their left side. Intraperitoneally administered 2-2-2 Tribromoethanol (Avertin; 0.5 mg/g dose; approximately 200 μL) 10 minutes before imaging was used to keep animals motionless during acquisition. Spectral fluorescence images were acquired of recumbent mice, using a Maestro fluorescence imaging system (CRi; Woburn, MA). The red filter set (excitation range 615 to 665 nm; emission, 700 nm longpass) was used to detect Alexa680-SPIO and the near infrared filter set (excitation range 710 to 760 nm; emission, 800 nm longpass) enabled detection of the cell membrane bound NIR815. Each spectral image set was acquired using a 5 sec exposure with acquisition at 10 nm steps through the emission range. The spectral fluorescence images consisting of the two dyes and the autofluorescence spectra were then unmixed based on their spectral patterns using commercial software (Maestro software, CRi). Line intensities were generated through the long axis of the spleen. Mean intensities of the signal at this organ and background were computed and averaged for each image.

5.5.7 - MR Imaging

Magnetic resonance imaging was conducted using a horizontal Varian 9.4 T small animal imaging system (Varian; Palo Alto, CA). Gradient coils were upgraded during the course of the study, going from a diameter of 14 to 12 cm and an applied field strength of
10 G/cm to 25 G/cm (both Varian). A dual coil, actively detuned system was employed; a 70 mm receiver volume coil was paired with a 2.5 cm surface coil (InsightMRI, LLC; Worcester, MA). Mice were induced using inhalation anesthesia, 4% isoflurane, and maintained for the duration of image acquisition with 2% isoflurane. The animals were placed on their right side in a split top mouse chamber sled (m2m Imaging; Cleveland, OH) affixed to a custom built poly-(methyl methacrylate) patient bed. The surface coil was applied to the left side of the animal and fixed in position using surgical tape to both the mouse and bed. Temperature and electrocardiography probes were used to monitor the rats in the 37°C environment supplied by an air heating system (SA Instruments, Inc.; Stony Brook, NY).

Scout scans were used to identify the anatomy of interest. Following recognition of the spleen axial sections, 1 mm thick, were acquired in a 36x36 mm field of view. Acquisition sets consisted of np=256, nv=256, under T₁-weighted and T₂*-weighted parameters. For the former, a spin echo multi-slice sequence was employed (2 NEX and TR/TE 2000/40 msec). T₂*-weighted scans were taken under two sets of conditions with increasing echo time (2 NEX, and TR/TE 200/6 (or 200/12) msec). Images were analyzed by defining an equal area ROI for the spleen and background (paraspinal) muscle. The relative intensity of the spleen was then calculated and statistical significance for the image analysis was p<0.05 using a two-tailed parametric analysis.

5.5.8 - Ex vivo Fluorescence Imaging

At each time point, following MR and in vivo fluorescent imaging, one subject per group was sacrificed. A necroscopy was performed to harvest the spleen, liver, lungs
and heart. Dual channel (700 nm and 800 nm) fluorescence images were acquired of the organs on an Odyssey flatbed laser scanning system (LICOR Biosciences; Lincoln, NE). After imaging was complete, organs were immediately fixed in 4% paraformaldehyde. The fixation of only one half of the spleen was performed (see below). The mean fluorescence of each organ was calculated. This value was determined by outlining each organ from the background using the trace thresholding tool in ImageJ. The mean intensities of each organ at each time point, for each group, were quantitatively compared.

5.5.9 - Immunohistology

The excised spleen was bisected prior to fixation (above) and the non-fixed half was embedded in Optimal Cutting Temperature medium (OCT, Tissue-Tek, Sakura Finetek Americas, Inc.; Torrance, CA) for cryostat sectioning. Sections (8 μm thickness) were initially coverslipped under cyanoacrylate ester glue and interrogated for GFP, NIR815 and Alexa680-SPIO by fluorescence microscopy or using the Odyssey in the 700 nm and 800 nm channels at 21 μm resolution.

The coverslip and glue were subsequently dissolved using an acetone bath for 48 hours which also served to cross-link the tissue. The same slide that had been imaged using fluorescence microscopy was then imaged for the presence and co-localization of GFP and nanoparticles. Nanoparticles were revealed by development of samples in Prussian blue stain. Here, a wash of a 1:1 mixture of 2% potassium ferrocyanide and 2% HCl for 20 minutes produces blue aggregates at sites of iron oxide (SPIO) deposits.
5.5.10 – Efficacy of Depletion

Peripheral blood lymphocyte (PBL) counts were determined by flow cytometry. PBL were obtained from mice at time of organ harvest, purified of red blood cells with ACK and stained with anti-CD19-FITC (1D3, rIgG2a) and fixed with 4% paraformaldehyde. Fluorescent populations (B cells) were assessed using a Guava Easycyte (Millipore).

The depletion of B cells in the spleen was determined in a similar fashion. A single cell suspension of splenocytes was obtained from the spleen of treated and untreated C57BL/6 mice (2 mice per group), 3 days after therapy. The number of B cells in the spleen was determined by labeling the splenocytes with anti-CD19 (APC) and measuring the population size on a FACSCalibur.
Chapter 5

5.6 - Results and Discussion

5.6.1 – SPIO Characterization and Cell Loading

Prior to imaging animals using fluorescently modified nanoparticles and dyes, the spectral characteristics of the cationic 53.5 nm superparamagnetic iron oxide nanoparticles derivated with Alexa 680 dye (SPIO) were measured. The concentration of Alexa 680 fluorophores per particle was determined from the difference in absorbance between labeled and unlabeled SPIO. A spectra of the nanoparticle absorbance normalized to that of an unmodified particle is shown in Figure 5.2A. Using a standard curve for nanoparticle concentration and the molar extinction coefficient of the dye (1840000 M/cm) the number of SPIO and of dye was determined for the absorbance sample. It was calculated that there were approximately 3 fluorescent molecules attached per particle.

Primary B cell activation was assessed ex vivo to determine if loading with 53.5 nm SPIO would have a negative impact on the cells' in vivo viability, distribution or function. Flow cytometry was performed on cells after incubation with particles for 1 or 24 hours and compared to non-labeled and phorbol ester (PMA) incubated B cells, Figure 5.2B. The SPIO did not appear to activate the cells as no increase in CD40, CD86, CD80 or MHCII was observed compared with that of unlabeled control, and expression was significantly less than activated with 1 μg/mL PMA.

Cells loaded with SPIO and NIR were examined by fluorescence microscopy to confirm their internalization and the prospects of their in vivo detection, Figure 5.2C-F. It was found that despite a high iron uptake of the cells (see below) that there was low
fluorescence from Alexa Fluor 680. It is speculated that this is due to a quenching of the dye due to its proximity to the iron oxide core of the particles. The labeling of NIR was rapid and robust, as detection of the cells was achieved with relatively short exposure time (200 msec).
Figure 5.2 - Fluorescence Labeling of Particles and Cells.

(A) Normalized absorption spectra of the dye-modified (green) and unmodified (red) SPIO. The peak at 680 nm is indicative of the presence of the dye. (B) Particles did not activate primary B cells, as determined by surface marker expression. (C-F) SPIO and dye loaded primary B cells were imaged, immediately after labeling. (C) White light, (D) Alexa 680 nm-SPIO, (E) NIR815 and (F) composite. Both contrast agents effectively labeled cells, however particle-core quenching of the Alexa dye limited its fluorescence. NIR815 membrane labeling was homogeneous and comparatively bright. All at 20x magnification.
MR imaging, initially using mouse cadavers, was carried out in order to develop the final imaging protocol described in Materials and Methods. Early live animal work involved both the intraperitoneal and tail vein injection of splenocytes loaded with SPIO and PKH26. Intraperitoneal injection as a means of administration was abandoned as migration of the cells to the secondary lymphoid tissues required days. From anecdotal references and initial imaging results, the slow accumulation of contrast in the spleen was difficult to verify. In contrast, tail vein injected cells were predominantly found in the spleen within 24 hours, as seen clearly seen below, in section 5.6.3.

Selection of sequence parameters, through which the $T_1$, $T_2$, $T_2^*$ or proton-density weighting could be achieved, was critical for the detection of the contrast agent used. For SPIO enabled nanoparticle detection of neuroinflammation in a rat model of pain (see Chapter 6) a $T_2^*$-weighted gradient echo multi-slice protocol had been developed. Using these sequence parameters (echo time; TE = 5.5 msec and repetition time; TR = 200 msec) as a starting point, values were varied in order to investigate effectiveness in imaging of SPIO-labeled B cells accumulated in the spleen, Figure 5.3. Increase in TR resulted in significant respiratory motion artifact. Increases in TE enhanced the $T_2^*$ effect slightly, however noise increased dramatically. Contrast, determined as the difference in mean signal between the spleen and background muscle (surrounding the spine), was assessed for increasing TE. It was found that increasing the TE above 6 msec did not provide greater splenic contrast. Use of a gradient echo multi-slice sequence with TR/TE of 200/6, 2 averages, 1 mm thickness and a resolution of 256x256 produced images with
a CNR of 82.25 for SPIO-B cell injected mice and only 31.67 for PBS injected mice; a CNR increase greater than two times.
Figure 5.3 - Preliminary MR and Histology.

(A, B) Representative image acquisitions in the sagittal plane of a mouse injected with SPIO-loaded B cells. The slices, 1 mm thick, were acquired with a gradient echo sequence with increasing TE as indicated at the base of each image. Right (rostral) to left (caudal) organs were identified; including the lungs (Lu), liver (Li), stomach (S), spleen (arrow and triangle) and kidneys (K) are clearly identified within an isointense region. Increasing TE produced greater signal decrease at the spleen. (C-H) Evidence of highly-efficient loading of SPIO-FITC and PKH26 into primary B-cells, following their injection and homing to the spleen. (D, G) PKH26 fluorescence was specific to the injected animals (562 nm emission). Likewise, (E) FITC channel signal was limited to autofluorescence in PBS injected as compared to (H) the SPIO-FITC loaded B cells.
5.6.3 - In Vivo Fluorescence Imaging

Preliminary work confirmed our ability to label primary B cells with both SPIO MR contrast agents and fluorescent membrane dye. During initial experimentation FITC and PKH26 were used. These fluorophores are non-ideal for in vivo imaging as they function at a highly tissue-attenuated region of the light spectrum. For in vivo imaging, SPIO were subsequently modified with Alexa 680 nm and the cells were membrane labeled with NIR dyes. Both dyes possess red-shifted excitation and emission profiles that enable greater tissue penetration (72). The excitation and emission maxima are listed for each of the fluorescent molecules in Table 5.3.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKH26</td>
<td>551</td>
<td>567</td>
</tr>
<tr>
<td>FITC(SPIO)</td>
<td>494</td>
<td>518</td>
</tr>
<tr>
<td>CellVue NIR815</td>
<td>786</td>
<td>814</td>
</tr>
<tr>
<td>Alexa Fluor 680(SPIO)</td>
<td>679</td>
<td>702</td>
</tr>
</tbody>
</table>

**Table 5.3 - Dye Suitability for In vivo Imaging**

Fluorescent molecules for preliminary (top half) and in vivo (bottom half) experimentation, according to manufacturers (MTTI and Invitrogen).

Using in vivo imaging-suitable dyes, it was possible to detect and monitor the distribution of B cells in vivo Figure 5.4. All four groups of animals were imaged in the near infrared red (NIR) channel using a Maestro system. Acquisitions in the deep red (Alexa-SPIO) channel were also obtained for groups that included SPIO loading. All mice were placed on a low-autofluorescence diet approximately one week prior to the
start of the study. This was done to limit background in the deep red channel. However, even with this precaution, the autofluorescence from (exposed) skin was still significant in the SPIO channel prior to injection of any contrast, Figure 5.4B. Further, the quenching of the Alexa Fluor 680 nm fluorescence, through proximity to the iron oxide core of the SPIO, limited the enhancement of signal following B cell administration. A similar effect had been noted using in vitro fluorescent microscopy (Figure 5.2).

Prior to injection of cells (day 0) the levels of fluorescence in the NIR channel were equal to that of background for all groups. Immediately following injection cells were visualized in the spleen, however there was generally high variability of signal intensity at this '1 minute' time point. The spleen was easily identified at 1 day, with little fluorescence observed elsewhere except at sites of the site of the tail vein injection. Here, it is speculated that a small fraction of the cells and dye may have been injected subcutaneously and thus required a much longer time to migrate to the spleen or disperse.
Figure 5.4 - In vivo Fluorescent Tracking of B Cells.

Representative whole animal fluorescence images of mice before and after injection of B cells loaded with SPIO and NIR contrast. Prior to injection (A-C), significant signal within the SPIO channel was evident at the dermis (B). Longitudinal imaging of this mouse, from group A (loaded with both NIR and SPIO; no knockdown), revealed rapid accumulation of the exogenous population in the spleen and at the sites of the tail vein injection (D-F). The signal decreased over time to the end time point of the study (J-L).
To determine the effect of the B cell depleting antibody, anti-CD79, on SPIO-labeled B cells the fluorescent signal intensity in the spleen was quantitatively compared across the four groups of animals, as outlined in Table 5.2. These groups were antibody-treated and untreated mice with B cells loaded with either SPIO and NIR or NIR-alone: group i) untreated, NIR and SPIO, group ii) treated, NIR and SPIO, group iii) untreated, NIR-alone, and group iv) treated, NIR-alone. The mean fluorescent intensity of the spleen and background and signal-to-background ratio (SBR) were then calculated for each animal (Figure 5.5). These data revealed that B cells localized to the spleen, within 1 day. In untreated animals that contained B cells membrane labeled with NIR alone, the signal gradually disappeared over the duration of the experiment (group D). For this group SBR retention at day 3 was 81.3% of day 1 levels. Untreated mice injected with cells loaded with both MR and fluorescent contrast (group i) also exhibited a gradual loss of signal with a SBR on day 3 that was 81.5% of day 1 levels. The continuing loss of signal over the remaining 11 days for the two groups was also similar.

In animals labeled with NIR-alone and treated with B cell depleting antibody (group iii) the loss of signal ratio was rapid, with only 41.5% of day 1 SBR ratio remaining by day 3. While SBR did not return to precontrast levels, likely due to some residual, non-cleared B cells, the signal continued to decrease gradually over the remainder of the experiment. However, for mice treated with the B cell depleting antibody following injection of SPIO and NIR loaded cells (group ii), SBR only decreased to 68% of day 1 levels at day 3. Continued signal ratio loss occurred on a rate comparable to that of the non-treated controls.
(A) For quantitative analysis, a user defined line of interest was placed on the (NIR channel) fluorescent image. (B) The signal intensity along the line was used to generate values for intensity of spleen signal and background. (C) The signal-to-background ratio, normalized to the day 1 value by group, demonstrated swift homing of contrast labeled B cells to the spleen. The ratio decreased gradually over the course of the experiment for non-knockedown groups (i and iv). In contrast, the metric decreased rapidly for the anti-CD79 depleted group containing B cells loaded with only NIR (iii). However, no depletion was observed in mice injected with cells loaded with both dye and SPIO, as measured by fluorescence (ii). Groups; i – untreated, NIR and SPIO, ii – treated, NIR and SPIO, iii – untreated, NIR-alone, and iv – treated, NIR-alone.
5.6.4 - MR Imaging

The biodistribution of B cells labeled with both SPIO and NIR was also observed by MR (Figure 5.6). Changes in relative signal normalized to day 0 (pre-contrast) between the spleen and back muscle changed significantly following injection with SPIO-loaded B cells (groups i and ii). Over the following two weeks, the normalized signal ratio gradually increased towards pre-contrast values. There was no statistically significant change (p>0.05) in the normalized intensity ratio for mice given cells labeled with NIR-alone (groups iii and iv). Thus, detection of the migration of the exogenous cell population to the spleen required the presence of SPIO. However, it was observed that the normalized signal ratio for the spleen gradually decreased following treatment of SPIO-free mice (group iii; NIR-only with anti-CD79 treatment). Possible changes to the MR T₂*-weighted signal of the spleen, through antibody mediated depopulation of the organ, could have such an effect over time. To our knowledge, this observation has not previously been presented in the literature regarding rituximab treatment and imaging.

Similar to the observations and measurements of in vivo fluorescence, in animals with SPIO-labeled B cells, relative signal changes were small between antibody treated and non-treated groups. There was an observed difference in signal intensity ratio at approximately 9 days following antibody injection. Nevertheless, for all other time points, differences between depleted and non-depleted mouse specimens that could be detected were non-statistically significant.
Figure 5.6 - MR Imaging of B Cell Distribution

(A-C) Representative axial T$_2^*$-weighted images of mice either pre-injection or on the indicated days following injection of SPIO-loaded B cells (group i). Signal in the spleen (arrow) decreased following cell introduction but gradually recovered over the duration of the experiment. (D) Relative signal intensity was calculated as the ratio of spleen and background muscle (an average) from user defined ROI. (E) Groups given SPIO-labeled B cells demonstrated rapid and pronounced relative signal changes normalized to pre-contrast (i and ii). There was no significant change in spleen signal for groups devoid of SPIO (iii and iv). Echoing the fluorescent data, there was also only limited difference in the normalized relative signal between treated (ii) and non-treated (i) groups that possessed SPIO-loaded B cells.
5.6.5 - Ex vivo Fluorescence

Consistent with findings from whole body fluorescent and MR imaging, the distribution of B cells as determined by ex vivo fluorescence indicated that homing to the spleen was rapid. The normalized mean fluorescent intensity (MFI) for each group, normalized by organ, is shown in Figure 5.7.

Homing of the re-administered B cells was almost entirely within the spleen, Figure 5.8A (non-normalized MFI). Some fluorescence in the liver, 1 day after cell injection, was also noted for all groups. In line with in vivo whole body fluorescent and MR imaging of the spleen, maximum signal was attained in the spleen (and all other organs) 1 day after injection and gradually decreased thereafter.

These organ specific fluorescent data also confirm that there is no significant difference between splenic signal in the treated and non-treated SPIO-labeled groups, Figure 5.8B. B cells were in some way inhibited from antibody-mediated depletion following intracellular loading of SPIO nanoparticulates. This effect was not a result of the B cell acquisition and purification protocol, as cells obtained and labeled with NIR-alone were robustly and rapidly depleted from the spleen.
Figure 5.7 - Ex vivo Fluorescence; By Group.

(A-D) The distribution of B cells by organ (spleen, liver, heart and lungs) for each group is shown, as determined by the mean fluorescence intensity (MFI) normalized to the day 1 intensity of each organ. For groups in which SPIO-labeled B cells had been administered to mice, there was a gradual decrease in fluorescence over two weeks (ii) with or (i) without introduction of antibody therapy. This paralleled the slow decrease in organs of signal from (iii) NIR-only labeled, non-depleted mice. A rapid decrease in MFI was measured after (iv) anti-CD79 therapy of NIR-only labeled B cells. Here, groups i, ii, iii, and iv correspond to A, B, C and D. (E) A representative false-coloured organ scan in the 800 nm (NIR) channel.
Figure 5.8 - Ex vivo Fluorescence; Absolute and Normalized Splenic Comparison.

(a) The non-normalized MFI, for group B, illustrates the predominance of spleen-targeted distribution. (B) Normalized MFI of the spleen alone, for each group, is plotted for the length of the experiment. Little difference was detected between the groups outside of antibody-treated, NIR-only (SPIO-free) group iii.
5.6.6 –Cytometric Analysis and Histological Correlation

Through longitudinal MR and fluorescent imaging it was apparent that there existed a discrepancy between the signal changes of treated animals from SPIO-and-NIR loaded (group ii) and NIR-alone labeled B cells (group iii). In order to verify that the anti-CD79 immunotherapy was effective towards the depletion of B cells, blood was obtained from a mouse at each time point, for each group. The number of B cells, as detected by fluorescently-labeled anti-CD19, was assessed relative to the total lymphocyte count, Figure 5.9A. Further, the splenic B cells depletion by antibody was also investigated, Figure 5.9B. It was apparent from these data that the anti-CD79 was indeed effective at reducing the number of B cells throughout the organism, if only for a time.
These figures indicate that the depletion of B cells was successful, using both a (A) global and (B-D) spleen-specific measure. (A) The peripheral blood lymphocyte quantification in untreated (i) and treated (ii) mice, into which SPIO and NIR loaded B cells had been administered. The arrow indicates the time of administration of immunotherapy (following day 1 image acquisitions). Within the spleen, B cell counts also decreased significantly following anti-CD79 therapy. (B) Unstained single cell suspension from spleen. (C) Anti-CD19 (APC) stained splenocytes from untreated animal. (D) Decreased number of B cells are detected following therapy. Average gated proportion of population for two mice 4 days after B cell introduction.
B cells were successfully being depleted by the immunotherapeutic treatment and not by sham PBS injection. This led to supplementary investigation of what role SPIO-loading had on the primary B cells, which apparently reduced their clearance, despite achieving this effect globally and within the spleen. Specifically, surface marker changes, as an indication of activation, were assessed to determine if the cells could be activated following loading by SPIO. LPS was used to activate the cells as a positive control. SPIO (non-fluorescently labeled, so as not to interfere with flow cytometry) were incubated with the cells under two conditions. The first was a recapitulation of the preliminary experiment to determine if the particles, when loaded during a two hour period, led to activation over the subsequent 24 hours (see section 5.6.1). To assess the ability of loaded B cells to become activated, SPIO were again loaded over 2 hours, but following washing away of excess particles, the cells were incubated with LPS for a 24 hour period. The results, shown in Figure 5.9, indicate that the SPIO do not inhibit the ability of the B cells to become activated, as assessed by surface marker differentiation.
Figure 5.10 – B Cell Function and SPIO Loading

Surface marker expression was assessed to determine the ability of B cells, following SPIO-loading, to activate. The blank group are primary splenocytes cultured for 24 hours in fully supplemented media. Activated cells were seen following incubation with LPS over 24 hours (0.1 µg/mL). Loading of cells with particles, SPIO (2h), again assessed the ability of SPIO to incidentally activate the cells. To determine if B cells could be activated following SPIO-loading, as used for the in vivo imaging experiments, labeled splenocytes were incubated with LPS for 24 hours, SPIO (2h LPS). The loaded cells appear to be able to undergo LPS mediated activation.
Histological analysis of the excised and axially sectioned tissue was also performed. These studies were undertaken in order to confirm that SPIO, NIR and GFP signal correlated within the spleen over the course of the anti-CD79-depletion monitoring experiment. Co-localization of the contrast agents with the exogenous cells is an important control to verify that the signal (or reduction in signal for MR) detected was not due to non-specific distribution of the agents outside of the cells following injection. For example, an initial concern was that anti-CD79 mediated depletion through ADCC processes would lead to the accumulation of originally-B cell loaded agents in macrophages.

In the initial examination, a relatively novel protocol was employed in order to examine fluorescent intensity data from all samples simultaneously, at the sub-organ scale (54). For our purposes, this allowed for the direct observation of splenic tissue fluorescence in all groups at multiple time points. Images of the NIR fluorescence indicated clearly that the signal intensity decreased as a function of time following loaded B cell injection. Disappointingly however, the lack of SPIO channel fluorescence made it impossible to directly correlate SPIO and NIR signal within the spleen. Fluorescence microscopy was also used to co-localize fluorescent signals, Figure 5.12. Despite high levels of autofluorescence in the GFP channel, correlation between sites of GFP, NIR and SPIO fluorescence could be made. Further, this signal was specific to GFP-B cell injected mice.
An application of moderately high-throughput histological laser scanning of splenic tissues. The samples were embedded in OCT and fresh frozen. Following axial (or axial-biased oblique) sectioning at 8 μm, the samples were placed directly onto an Odyssey laser scanner. With a resolution of 21 μm, it is possible to discern with moderately-high-resolution the fluorescent signal from the spleen. (A-D) (A) No NIR signal is present in control tissues (mice without B cell injection). The decrease of NIR signal (800 nm channel) of group i samples (NIR- and SPIO-loaded) from (B) day 1 through (C) day 5 and finally, (D) day 15. All images have identical brightness and contrast values. The flatbed scanning profile allows for acquisition of numerous samples at the same time, lower magnification (F).
Figure 5.12 – Histological Co-localization.

(A-D) Representative images of the spleen of a mouse without administration of B cells. (A) Despite the absence of any green fluorescent protein, there is still considerable fluorescence in this low wavelength channel. There is little detectable cellular signal in the (B) SPIO or (C) NIR channels, as shown on (D) the composite image. (E-F) Representative images of the spleen an untreated mouse 7 days after injection of B cells loaded with both SPIO and NIR, group i. (E) Despite the comparable background autofluorescence in the GFP channel, there were distinct, punctate areas of increased signal. These localized well with the (F) SPIO and (G) NIR fluorescence channels. The composite image shows that indeed there is considerable overlap of all three channels (arrows), apart from the background autofluorescence. All images taken at 40x magnification.
5.7 - Conclusion

The immune system is a dynamic and highly coordinated assemblage of proteins and cell types that protect the body from foreign invaders. Lymphocytes are the mediators of adaptive immunity and are able to migrate throughout the body as well as to specific sites of the lymphatic system including the thymus, lymph nodes and spleen. In order to analyze this complex series of events, procedures are required that can simultaneously track the migration of lymphocytes into different tissues. This is especially relevant when the system operates in an abnormal fashion.

Autoimmune disorders, such as systemic lupus erythematosus present a host of clinical manifestations that impact quality and length of life (73, 74). Successful application of B cell depleting antibody, which has been shown to be effective in treatment of non-Hodgkin's lymphoma (1) and rheumatoid arthritis (75), to SLE has been elusive (9). Understanding the distribution of autoimmune disorder relevant populations, such as B cells, prior to and following attempted therapy would permit the determination of effectiveness of treatment with respect to duration and extent. Here, we investigated the use of non-invasive cellular and molecular imaging approaches for the repeated measurement of B cells longitudinally.

We have shown the ability to effectively load superparamagnetic iron oxide nanoparticles (SPIO) into lymphocytes (69). This was accomplished without the use of potentially toxic exogenous agents (76-78). The 53.5 nm diameter aminated and cross-linked dextran-coated SPIO nanoparticles were able to effectively label non-phagocytic lymphocytes and showed significant $T_2^*$-weighted contrast. When used to label primary murine B cells no significant activation, as determined by surface marker expression, was
noted. Cells labeled with these fluorescently-labeled agents could be identified under fluorescent microscopic observation, however the brightness of the fluorophore used, the Alexa Fluor 680 nm dye, was apparently quenched by the iron oxide core of the nanoparticle to some degree. Due to this effect, and a desire to have multimodal cell tracking capabilities, a second cellular marker was introduced, the CellVue NIR 815 nm membrane labeling dye (NIR). With NIR we demonstrated the ability to effectively label the primary B cells with the required concentration to enable in vivo fluorescent detection.

Contrast loaded B cells homed quickly and specifically to the spleen. This was determined non-invasively in all groups by a specific signal increase in the spleen by fluorescence imaging. Here, a signal-to-background ratio of approximately 30 times was recorded for all groups. Hypointensity of the spleen in MR T₂*-weighted imaging for SPIO and fluorescently labeled cells confirmed a multimodal capability to detect the presence of an introduced cell population. Relative to pre-injection values, there was an approximate 40% change in normalized signal intensity ratio 1 day after cellular administration. This correlated with fluorescent signal increases in both ex vivo imaging techniques, organ laser scanning and histological evaluation.

For mice in groups that were treated with PBS there was a gradual loss of MR and fluorescent signal (in vivo and ex vivo) over time. By the final imaging time point, MR signal ratio had returned to within statistical error of the pre-injection values. Similarly, after 15 days only 40% of the fluorescent signal (determined by signal-to-background ratio) remained in the spleen relative to day 0 levels.
In treated groups, significant differences in the change in signal following treatment were seen between the MR-and-NIR and NIR-alone loaded lymphocytes, under all modalities of imaging. MR contrast generation in the spleen had been found to be specific for SPIO loaded cells; no contrast developed between the spleen and background muscle when lymphocytes with NIR-alone had been introduced. However, there was no significant change in signal intensity ratio between the two SPIO-loaded B cell groups; PBS and anti-CD79 treated. Using fluorescence whole body imaging, these results were essentially recapitulated. It was seen again that there was little difference in the splenic signal between the two SPIO-loaded groups.

These results were quite distinct from the fluorescent spleen intensity over time in animals injected with B cells that had only been label with NIR dye. Unlike the gradual loss of signal trending towards the pre-injection level, a rapid decrease in signal-to-background was observed in anti-CD79 treated mice. These results were confirmed by ex vivo organ and histologically prepared tissues for fluorescent measurements. It was found that signal from the organs, once resected, reported similar outcomes. Namely, the fluorescent intensity was specific to the spleen and decreased rapidly only for the anti-CD79 treated NIR-only loaded mice.

Flow cytometry of blood at terminal imaging time points was collected in order to determine the effectiveness of antibody treatment in depleting circulating B cells. From these data it was seen that depletion therapy was successful in removing a significant proportion of the circulating B cell population. This outcome was seen in both treated groups (dual-labeled and NIR-only), while the peripheral blood lymphocyte values varied little for the PBS injected animals. Further, splenic B cell quantification, also by flow
cytometry, indicated that the number of B cells decreased significantly following treatment, as compared to untreated control. With the knowledge that the antibody was indeed effective upon administration it was apparent that the SPIO nanoparticles were in some way influencing the depletion of the labeled, introduced B cells in the location to which they had accumulated, the spleen. Determination of the biochemical or physical means of this inhibition is extremely difficult. It is impossible to mimic the specific and complex microenvironment of this organ ex vivo. This would be required in order to discern the role that each chemical, mechanical and biological factor may have in the therapeutic efficacy of the antibody mediated depletion. However, in an initial attempt to determine the possible effect of SPIO loading on B cell function, a post-loading activation study was presented. Here, it was shown that B cells can be activated by LPS as determined by surface marker expression in flow cytometry.

These results indicate clearly that detection and tracking of an introduced population of B cells is possible using SPIO nanoparticles. However, the outcome of little apparent signal ratio change in either MR or optical modalities indicates that the approach of ex vivo loading these contrast agents are inadequate to the task of monitoring therapeutic efficacy. This is a particularly interesting conclusion from the standpoint of the application of nanomaterials in biological, and especially biomedical, settings. It also serves to highlight a concern for the use of MR approaches to track lymphocytes in a similar fashion to the extant drawbacks associated with radiotracer and optical approaches.

In a recent study comparing bioluminescent and SPIO approaches to non-invasive cell tracking, it was found that the SPIO used exhibited an extended residence lifetime in
vivo, regardless of the presence or state of the cells they were supposedly meant to track (79). This led to the conclusion that bioluminescent proteins were a better marker of myocardial cell survival kinetics than MR through SPIO. We have found that the change in signal from MR and optical techniques do not mimic the distribution profile of cells tracked without SPIO. It should be noted that the present study’s findings do not correlate exactly with the recently published work finding fault with SPIO. We were able to determine a co-localization of SPIO, NIR and B cells. And in fact, others have indeed shown that SPIO and bioluminescence are both effective imaging approaches to determine the presence of stem cell transplants to injured cardiac tissue (80).
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5.8 - References


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CHAPTER 6. Comparative Analysis of Nanoparticle-Antibody Conjugations: Carbodiimide Versus Click Chemistry

6.1 - Abstract

Ex vivo labeling of contrast agents into cells affords high levels of loading for subsequently sensitive in vivo detection. However, as we have shown in the previous chapter, the ex vivo loading of superparamagnetic iron oxide (SPIO) nanoparticles into B cells inhibited the cells ability to be depleted using an antibody therapy. In vivo targeting of cells offers an alternative approach; wherein the cells are labeled in vivo using systemically delivered particles. While achieving lower contrast delivery per cell, these methods still allow for non-invasive detection. The major advantage of this strategy applied for the monitoring of B cell response to immunotherapy would be to avoid the onerous requirements of extracting, purifying and loading B cells, and their possible dysfunction as a result.

Typically, in vivo applications require functionalizing the nanoparticles directly with targeting agents. The ability to modify the physical, chemical, and biological properties of nanoparticles (NPs) has led to their widespread use as multi-functional platforms for drug delivery and diagnostic applications. Antibodies (Ab) remain an
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attractive choice as targeting agents because of their large epitope space and high affinity; however, implementation of Ab-NP conjugate strategies are plagued by low coupling efficiencies and the high cost of reagents.

Click chemistry may provide a solution to the problem of Ab-NP probe manufacture with reported coupling efficiencies nearing 100%. While click chemistries have been used to functionalize nanoparticles with small molecules, they have not previously been used to functionalize nanoparticles with Ab. Concerns associated with extending this procedure to Ab is that reaction catalysts or the ligands required for cross-linking may result in loss of functionality. Here, we document the evaluation of the efficiency of conjugations between Ab and superparamagnetic iron oxide NPs using click chemistry. Results were compared with conjugates formed through carbodiimide (EDC) cross-linking. The click reaction allowed for a higher extent and efficiency of labeling compared with EDC thus requiring less Ab. Functional assessment of these probes in an in vitro cell labeling schema has also been performed. In comparison to EDC, conjugates prepared via the click reaction exhibited improved binding to target receptors.
Nanoparticles (NPs), commonly defined as organic or inorganic materials with at least one length dimension below 100 nm, are being widely investigated as drug delivery vehicles and/or imaging agents. Interest in NPs largely stems from their ability to carry a large therapeutic payload (or ample amounts of contrast agent), the ability to finely tune physicochemical properties, which can influence their pharmacokinetic and pharmacodynamic profiles, and the ability to functionalize their surface with molecularly specific targeting agents. For both diagnostic (1) and therapeutic (2) purposes, including immune cell relevant applications, it is becoming increasingly recognized that specific targeting of NPs is critical towards their effective use.

Ex vivo loading of a particulate agent is a proficient means to achieve high intracellular payloads of contrast or therapeutic agents. An example of this approach was detailed for in vitro (Chapter 4) and in vivo (Chapter 5) applications. However, this method requires that the cells of interest be removed, often quite abrasively from an organism or patient, for example in the case of splenocyte purification. Such an approach is well suited for some applications, but not in situations when invasive attainment of a cell population would be detrimental to its further study.

In vivo targeting of NP is a more sophisticated approach wherein agents are directed with some engineered-specificity towards cells of interest. When targeted, reduced amounts of therapeutic and imaging agents are required compared with systemically delivered non-specific vehicle (3). Furthermore, targeting greatly reduces nonspecific background signal from accumulation of contrast agents at undesirable sites.
when compared to non-targeted NP. Together, lower dose and greater specificity reduce undesirable toxicity and lead to improved efficacy of therapeutics.

Superparamagnetic iron oxide nanoparticles (SPIO) are a widely used NP system affording $T_2^*$-weighted contrast for magnetic resonance (MR) imaging applications (4). Recently, SPIO have been conjugated to a variety of targeting ligands to provide cellular and molecular specificity for in vitro diagnostic (5) and in vivo imaging (6) applications. Strategies for targeting SPIO most often include the use of peptides, endogenous ligands and monoclonal antibodies (7-9).

Despite their relatively large size (roughly 150 kDa) antibodies remain an attractive choice because of their combinatorially large epitope space (of approximately $10^{15}$) and their high affinity for targets with $K_d$’s often on the order of nM (10). Antibodies also have the additional advantage of being widely pursued for use in pre- and clinical applications for the therapy of prevalent disorders. Antibody therapies for cancer such as Trastuzumab (targeting Her2/neu) (11), for inflammatory disorders such as Infliximab (an anti-TNF antibody) (12) and antiviral antibodies such as Palivizumab (targeting respiratory syncytial virus) (13) are used to study and treat disease globally.

Currently, one of the greatest obstacles facing the use of NP-antibody conjugates is the low efficiency of Ab coupling to NPs, which can result in the need for large quantities of antibody and prohibitive costs (14). One example of a common approach used for conjugation of antibodies to NPs involves carbodiimide cross-linking (15). The zero length cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) reacts with carboxylated NPs in the presence of sulfo-N-hydroxysuccinimide
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(sulfo-NHS) to form amine-reactive sulfo-NHS esters. Subsequent addition of antibodies results in coupling between the NPs and primary amines on the antibody via a stable amide bond. Unfortunately, the reaction is non-ideal as it is highly inefficient and thus requires a high excess of antibody. Typically, only about 1-20% of the antibody used during the conjugation procedure will be coupled to the NP (9, 16, 17).

Click chemistry refers to modular chemical conjugations with an emphasis on simple reactions that can take place under a range of conditions with stereospecificity and high efficiency (18). Recently, the emergence and adoption of the click chemistry philosophy has had a large impact in drug discovery and materials synthesis. The rapid rise of click chemistry is evident from its novel use in a variety of disciplines including medicinal chemistry (19), materials and polymer science (20, 21) and molecular imaging (22, 23). The most widely adopted reaction of this type, the Cu\(^{1}\)-catalyzed terminal alkyne-azide cycloaddition (CuAAC), was developed by the Sharpless and Meldal groups independently in 2002 (24, 25).

Previously, it has been shown that the coupling of low molecular weight species such as peptides (26) and fluorophores (27) to SPIO can be accomplished with CuAAC. However, evidence that functional antibodies can be coupled to SPIO through CuAAC is lacking. Likewise, reference of large protein linkage to any NP system using a click approach is scarce. Gupta et al. have briefly described holo-transferrin bound viral capsids, although this was reported by way of TEM (transmission electron microscopy) micrographs (28). Lipases have been conjugated to gold nanoparticles and retained their function (29), however a major concern with using CuAAC for NP-antibody coupling
reactions is that the antibodies will be irreversibly degraded and/or modified during the reaction. For example, it has previously been shown that the CuAAC reaction can result in the irreversible degradation of viral capsids (30) and nucleic acids (31). Further, antibodies could become non-functional due to modification of the innate protein with click reactive ligands.

In this work we evaluated the use of CuAAC for conjugation of SPIO to anti-CD20. Anti-CD20 is a clinically approved chimeric monoclonal antibody that has been therapeutically approved for use in treating B cell lymphoma. This antibody has also been used previously to target SPIO to CD20 expressing tumors (32). We have sought to comparatively assess the utility of the CuAAC reaction for antibody targeting of SPIO against an accepted method, that of carbodiimide conjugation. Particle labeling was assessed for both strategies by means of the efficiency and extent of protein-to-particle labeling and by functional cell-targeting assays. It is our expectation that determination of best practices for Ab-NP production will significantly impact their ability to be applied in biomedically relevant situations.
6.3 - Experimental Procedures

6.3.1 - Materials

Azido-dPEG$_{12}$ NHS ester and Propargyl-dPEG NHS ester were purchased from Quanta BioDesign Ltd., (Powell, OH). The nanoparticle coating material, dextran T10, was purchased from Amersham Biosciences (now GE Healthcare, Piscataway, NJ). Reagent grade IgG (Immunoglobulin G) from rat serum was acquired from Sigma Aldrich (St. Louis, MO), while anti-CD20 antibody, Rituximab, (Genentech, South San Francisco, CA) was a gift of the Eisenberg group. Human lymphoma B cells (Burkitt GA-10) were obtained from ATCC (Manassas, VA). Bathocuproinedisulfonic acid (BCS) was acquired from Acros Organics (Geel, Belgium). All other reagents were purchased from Thermo Fisher Scientific (Waltham, MA), unless otherwise noted.

6.3.2 - SPIO Synthesis and Amination

SPIO were prepared by chemical co-precipitation, as previously described in Chapter 3 and reference (33). Briefly, 0.7313 g FeCl$_2$ and 1.97 g FeCl$_3$ were each dissolved in 12.5 mL diH$_2$O and added to 25 g dextran T10 in 50 mL diH$_2$O at 4°C. Ammonium hydroxide (15 mL) was slowly added to this mixture turning the light yellow colored solution black. This nanoparticle slurry was then heated to 90°C for 1 h and cooled overnight.

Purification of SPIO was accomplished by ultracentrifugation of the mixture at 20K RCF (relative centrifugal force) for 30 min. Pellets were discarded and the
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supernatant was subjected to diafiltration against greater than 20 volumes of 0.02 M citrate, 0.15 M sodium chloride buffer, using a 100 kDa cutoff membrane filter (GE Healthcare). The purified particles were then cross-linked by reacting the particles (10 mg Fe/mL) with 25% (v/v) 10 M NaOH and 33% epichlorohydrin. After mixing for 24 hours, the particles were briefly dialyzed and then functionalized with amines by adding 25% ammonium hydroxide. This reaction was allowed to continue for another 24 h followed by diafiltration as above.

6.3.3 - SPIO Characterization

The hydrodynamic diameter of the nanoparticles was measured using a Zetasizer Nano-z (Malvern Instruments, Malvern, UK) through dynamic light scattering (DLS). SPIO particles were diluted in PBS to a concentration of approximately 0.5 mg Fe/mL and read in triplicate. The values reported for all samples are the intensity peak values.

The longitudinal ($R_1$) and transverse ($R_2$) relaxivity of each particle was calculated as the slope of the curves $1/T_1$ and $1/T_2$ against iron concentration, respectively. $T_1$ and $T_2$ relaxation times were determined using a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz). $T_1$ measurements were performed by collecting 12 data points from 5.0 to 1000 msec with a total measurement duration of 1.49 minutes. $T_2$ measurements were made using $\tau = 1.5$ msec and 2 dummy echoes, and fitted assuming a monoexponential decay.

The number of amines per particle was determined following the general procedure described by Zhao et al. (34). Briefly, iron oxide particles at a concentration of
2 mg/mL Fe were reacted with excess N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Calbiochem, San Diego, CA) for 4 hours. SPIO were washed of excess SPDP through repeated precipitation in isopropanol and resuspension in PBS. The particles were then run through a 50kDa MWCO centrifugal filter (YM-50, Millipore, Billerica, MA) either with or without the addition of the disulfide cleavage agent (tris(2-carboxyethyl)phosphine) (TCEP). The difference of the absorbance of these two samples at 343 nm was used to determine the concentration of SPDP in the filter flow. Adjusting for dilution, the number of amines per particle was determined.

6.3.4 - FITC Modification of SPIO

Fluorescein-isothiocyanate (FITC) was used to fluorescently label amine-functionalized cross-linked SPIO by reacting at a molar ratio of 19:1 FITC to iron. The unbound FITC was washed from the FITC-SPIO using a PD10 gel filtration column (GE Healthcare) equilibrated with phosphate buffered saline (PBS). All particles were FITC-labeled prior to subsequent modification, ensuring that all particles had equal fluorescent labeling.

6.3.5 - Carboxylation of SPIO

A schematic outlining the carbodiimide cross-linking procedure is illustrated in Figure 6.1A - strategy i. FITC labeled, amine-functionalized NPs (as shown in Figure 6.1B) were derivatized to acid residues following reaction with an excess of succinic
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anhydride in basic solution. Specifically, 200 μL of NH$_2$-SPIO (5 mg Fe/mL) in 0.02M citrate buffer, pH 8 was added to 10 μL of 1 M NaOH followed by 10 μL of 4 M succinic anhydride in DMF. The reaction was allowed to mix for 4 hours. Carboxylated-SPIO (COOH-SPIO) were subsequently precipitated three times in 4 volumes of isopropanol to remove free reactants.
Figure 6.1 - Schematic of Antibody-SPIO Conjugation Strategies.

(A) Fluorescently labeled, amine functionalized SPIO were reacted along one of two routes to produce antibody-SPIO conjugates. In strategy (i) carbodiimide chemistry is utilized, following the conversion of the surface amines to COOH groups utilizing succinic anhydride. In strategy (ii), antibody-labeled SPIO are produced by employing Cu₁-catalyzed terminal alkyne-azide cycloaddition (CuAAC). Azide-modified SPIO are “clicked” to alkyne-labeled antibodies in the presence of copper. (B) Schematic illustrating the preparation of FITC-SPIO. (C) Schematic illustrating the alkyne-PEG modification of antibody, which was necessary for the CuAAC reaction.
6.3.6 - Carbodiimide Conjugations

Carbodiimide coupling reactions were accomplished with FITC labeled COOH-SPIO in 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0. To 50 μL of SPIO (2 mg Fe/mL), 10 μL each of 200 mM EDC and 500 mM sulfo-NHS was added. Solutions mixed for one hour at 25˚C, were then precipitated in 500 μL of isopropanol. A resuspension of the NPs in 100 μL of 10 mg/mL antibody solution in PBS, pH 7.4 was allowed to react at 15˚C overnight. Purification was carried out using a MS magnetic column in PBS.

The BCA Protein Assay was used for protein concentration of conjugate solutions, after correcting for background with unlabeled SPIO. To determine number of protein per particle, the molarity of SPIO was assessed spectrophotometrically by dissolving the NPs and oxidizing the iron with 6M HCl and 3% H₂O₂. Absorbance at 410 nm was compared to a standard curve and particle concentration was calculated assuming 8924 Fe atoms per particle, as previously determined (33).

6.3.7 - Azide Modification of SPIO

A schematic outlining the CuAAC cross-linking procedure is found in Figure 6.1A - strategy ii. FITC-SPIO was reacted with the amine-reactive azido-dPEG₁₂ NHS, diluted 10 times from stock in dimethyl sulfoxide (DMSO), in 0.1 M sodium phosphate buffer, pH 9. The linker was added at 100 times molar excess to the SPIO. After mixing for 4 hours, the SPIO were purified twice on PD10 columns equilibrated with PBS.
Azide-SPIO was then concentrated on an Ultracel 30k centrifugal filter (Millipore, Billerica, MA) in PBS.

6.3.8 - Alkyne Functionalization of Antibodies

Alkyne functionalization of antibodies, for both IgG and Rituximab as seen in Figure 6.1C, was accomplished by addition of 10% v/v propargyl-dPEG-NHS in DMSO to 10 mg/mL antibody in 0.1M sodium phosphate buffer, pH 9. The concentration of propargyl-dPEG-NHS was varied to provide different degrees of labeling per antibody. Antibody was purified on a PD10 column equilibrated with PBS and then re-concentrated using Ultracel 30k filters. Antibody concentration post-purification was assessed spectrophotometrically at 280 nm using a molar extinction coefficient of 210000 M⁻¹cm⁻¹.

6.3.9 - CuAAC Conjugation

SPIO was conjugated to alkyne functionalized IgG and anti-CD20 using the same procedures. FITC labeled N₃-SPIO (3 mg Fe/mL) was mixed with varying volumes of 10 mg/mL alkynated-antibody, 5 mM BCS, 1 mM CuSO₄ and 5 mM sodium ascorbate. Final volumes of the reactions were brought to a constant level with the addition of PBS. The samples were mixed for 4 h at 15° C. Samples were cleaned of the reaction additives using an YM-50 spin column. The antibody-linked nanoparticles were then purified from unbound protein on a MS magnetic column (Miltenyi Biotec, Bergisch Gladbach, Germany). Protein per particle labeling was determined as above.
6.3.10 - Cell Labeling and Assessment

SPIO were incubated with 100 μL of 1x10$^6$ cells/mL Burkitt’s GA-10 lymphoma B cells for 30 minutes at 37˚C, 5 % CO$_2$ in a 96 well plate. SPIO were added at final concentrations of 50 μg Fe/mL and 10 μg Fe/mL for carbodiimide- and click-conjugated SPIO, respectively. In the antibody inhibition experiments, 50 μg/mL (final concentrations) of free anti-CD20 was added prior to the addition of the antibody-conjugated nanoparticles. The free, unbound particles, were purified from the cells through three PBS washes at 1k RCF for 5 minutes each. Cells were finally resuspended in 300 μL of PBS and placed in a 96-well plate to be read using a Guava Easycyte Plus system (Guava Technologies, Hayward, CA). Flow cytometry data was analyzed using FlowJo (TreeStar Inc., San Francisco, CA).

Click-conjugated particles at a concentration of 50 μg Fe/mL were incubated with cells as above, using 30x10$^3$ cells. Images were acquired with an Olympus IX 81 inverted fluorescence microscope using a LUC PLAN 40x objective (NA 0.6; Olympus) following fluorescein excitation by and an X-cite 120 excitation source (EXFO, Quebec, QC). Micrographs were taken using a back-illuminated EMCCD camera (Andor Technology PLC, Belfast, Northern Ireland).
6.4 - Results and Discussion

6.4.1 - Antibody Modification

Antibodies were coupled to SPIO using either conventional carbodiimide chemistry or CuAAC, as shown in Figure 6.1A. The SPIO used throughout the present study possessed an average hydrodynamic diameter of 33.4 nm and $R_1$ and $R_2$ values of 13.56 mM$^{-1}$sec$^{-1}$ and 71.00 mM$^{-1}$sec$^{-1}$, respectively. The SPIO were also aminated (~185 NH$_2$ per particle), making this probe well suited to modification with dye and proteins. Initially, all CuAAC conjugations were carried out using rat IgG so that the reaction parameters could be optimized in a cost-effective manner. Further, FITC dyes were bound to the particles prior to any subsequent protein modification. This served to eliminate interference of fluorescent labeling by proteins or click-ligands at a later stage.

A series of biochemical cross-linking experiments were performed in order to determine the optimal binding efficiency of antibody to SPIO using the CuAAC strategy. The first parameter to be varied was the number of alkyne groups introduced onto IgG. This was accomplished by varying the labeling ratio of alkynating reagent, CH-PEG-NHS, to IgG from 15:1 to 100:1. In a separate reaction, azide-labeled SPIO were prepared by reacting aminated SPIO with a 100-fold molar excess of N$_3$-PEG-NHS, as seen in Figure 6.2. Following appropriate purification procedures, alkyne-labeled IgG were coupled to the azide-SPIO through CuAAC in a catalytic copper solution. For this reaction, the molar ratio of IgG per SPIO was held constant at 17.5:1. After a 4 hour incubation, unbound IgG were removed by magnetically purifying the SPIO and the average number of IgG coupled to each SPIO was quantified. It was found that the
number of IgG per SPIO increased with the degree of alkyne incorporation, to a maximum labeling of 10-13 IgG per SPIO for this series of reactions. The maximum number of IgG per SPIO was achieved when IgG was reacted with >35-fold molar excess of CH-PEG-NHS. Therefore, all subsequent alkynating reactions were carried out utilizing these lowest saturating conditions.

It can be seen that there is significant variability in the reaction as we approach the saturation labeling plateau. This is a result of cross-linking of N₃-SPIO and CH-IgG into large aggregates. The occurrence of this phenomenon was difficult to overcome because purification of excess protein is required in order to assess how much remains bound. Purification is not possible when the particles are no longer soluble. It was found that aggregation could be minimized through application of several careful steps during and after the reaction.

Specifically, immediate mixing of all reactants after each was added helped reduce aggregation. The order of addition was not important, however it was kept constant. Additional PBS was added prior to any purification, which appeared to aid in preventing the loss of all material if any precipitate had formed. Further, as noted in the materials section, a filtration step was added prior to the magnetic purification. It appears counterintuitive that sedimentation would help particles remain in solution, but it has been found that aggregation in solution is more likely to result in strong column binding during the magnetic purification step (even after removal from the magnet). Therefore, removal of such material (which will form a film on the filter), as well as excess reactants, prior to application to the magnetic column was beneficial.
Figure 6.2 - Effect of IgG alkylation on conjugation to SPIO.

To determine the minimally required alkyne residues on IgG necessary to achieve maximum conjugation with SPIO, IgG was reacted with increasing amounts of CH-PEG-NHS. The alkyne-IgG samples were then “clicked” to N3-SPIO and the degree of labeling was assessed. The degree of antibody labeling began to level off at an approximate starting labeling ratio of 35 CH-PEG-NHS:IgG.
6.4.2 - Saturation Labeling

An important reported advantage of pursuing CuAAC reactions has been the high efficiency of conjugations. This is a particularly desirable property when procedures involve large and expensive proteins such as antibodies. As such, we next determined the conjugation efficiency between IgG and SPIO for labeling ratios of alkyne-IgG to azide-SPIO ranging from 2.5:1 to 35:1. As shown in Figure 6.3, there was nearly a 100% coupling efficiency when the labeling ratio was less than ~20 IgG per SPIO. At higher labeling ratios, the number of IgG per SPIO did not improve, likely because there was insufficient space on the SPIO surface for additional conjugations. Utilizing this information, subsequent conjugation of the clinically approved anti-CD20 was materially conserved, as a labeling ratio of only 15 antibodies per SPIO was used.
The click reaction between protein and SPIO was performed with increasing molar excess of IgG to SPIO. The labeling efficiency was ~100% for all conjugations up to a labeling ratio of 20 IgG per SPIO. Higher labeling ratios did not result in any increase in the number of conjugated antibodies.
6.4.3 - Bioconjugation Comparison

To this point all reactions had seen rat IgG coupled to FITC labeled SPIO by CuAAC. Prior to assessment of whether antibodies that have been coupled to SPIO remain functionally active following the CuAAC reaction, antibody (anti-CD20) was coupled to SPIO using CuAAC. For comparison, the same antibodies were also coupled to SPIO using carbodiimide chemistry. Each step of the carbodiimide reaction was conducted under saturating conditions to maximize the conjugation efficiency. Specifically, carboxylated SPIO were reacted with an excess of EDC and sulfo-NHS in MES buffer, pH 6.0 for 1 hour in an attempt to achieve complete activation of all available carboxyl groups. To minimize subsequent hydrolysis of the NHS-activated SPIO, unreacted cross-linking agents were removed as rapidly as possible (~2-5 minutes) by precipitating the SPIO with isopropanol. The SPIO were then resuspended directly in an excess of anti-CD20 in PBS, pH 7.4 and mixed overnight.

As shown in Figure 6.4A, the copper catalyzed ligation of anti-CD20 to SPIO after 4 h yielded approximately 6.85 anti-CD20 per SPIO. Carbodiimide cross-linking only resulted in 2.77 anti-CD20 per SPIO. The labeling efficiency for each conjugation strategy, defined as a percentage of conjugated protein per SPIO over total protein per SPIO is presented in Figure 6.4B. The click chemistry reaction achieved 45.6% efficiency, while the carbodiimide reaction resulted in a labeling efficiency of only 8.3%. It was surprising that despite a relatively high degree of labeling by the protein through CuAAC, this was significantly less than achieved with rat IgG.
It is hypothesized that the reduced efficiency may stem from a lower incorporation of alkyne onto the anti-CD20 compared with IgG, perhaps due to subtle differences between the proteins. Nonetheless, CuAAC still resulted in a 5.5-fold improvement in the efficiency of labeling compared with carbodiimide chemistry.
Figure 6.4 - Comparison of SPIO Labeling with Anti-CD20 Using CuAAC and Carbodiimide Conjugation Procedures.

(A) Extent of reaction as measured by final number of antibody per SPIO. Following the optimization of IgG-SPIO conjugation using CuAAC, anti-CD20 antibodies were “clicked” to SPIO. The average number of antiCD20 per SPIO was determined by BCA Protein Assay to be 6.85 molecules per particle. Labeling of SPIO by anti-CD20 was also accomplished using the classic carbodiimide method; however a lower final labeling ratio of 2.77 antibodies per SPIO particle was achieved, despite using more antibodies during the conjugation procedure. (B) Percent efficiency of reaction by conjugation method. Here, the ratio of final protein per NP with respect to the initial value has been plotted as a percent. For the CuAAC-mediated cross-linking of anti-CD20 the reaction approached 50% efficiency, while the EDC conjugates reached almost 10%.
6.4.4 - Functional Application

Binding of anti-CD20-conjugated SPIO to CD20-positive B cells was assessed by flow cytometry. All SPIO were fluorescently labeled prior to the respective conjugation protocols to enable fluorescence detection. The same fluorescently labeled SPIO were used for both the CuAAC and carbodiimide reactions to ensure that total cellular fluorescence could be correlated to the extent of SPIO binding. Thus, it was possible to quantitatively compare the equally fluorescent EDC- and CuAAC-particle strategies.

As shown in Figure 6.5 (top row), it was found that at a concentration of 10 μg Fe/mL, anti-CD20-targeted SPIO prepared using click chemistry could be used to successfully target B cells. Specificity was confirmed by competitively inhibiting SPIO binding using an excess of free anti-CD20 antibodies. Surprisingly, anti-CD20-targeted SPIO prepared using carbodiimide chemistry did not result in any appreciable B cell labeling using equivalent concentrations (10 μg Fe/mL; D-F). In fact, similar levels of B cell labeling could only be achieved when the iron concentration was increased to 50 μg Fe/mL (Figure 6.5, bottom row). Again, for both concentrations of the carbodiimide-conjugated targeted nanoparticles, specificity was confirmed by competitively inhibiting SPIO binding using an excess of free anti-CD20 antibodies. This data suggests that the anti-CD20-labeled SPIO prepared using click chemistry have a higher affinity for the CD20 receptors on B cells compared with analogous nanoparticles prepared using carbodiimide chemistry. It is likely that this increase in affinity is due to the greater number of anti-CD20 per particle.
Functional targeting of SPIO to B cells was accomplished for click- and carbodiimide-cross-linked anti-CD20-SPIO conjugates. Top row shows the flow cytometric analysis of B cells that were incubated with 10 μg Fe/mL of CuAAC conjugated (A) IgG-SPIO, (B) anti-CD20-SPIO, and (C) anti-CD20-SPIO in the presence of excess free antibody. Labeling of cells with the EDC conjugated particles (10 μg Fe/mL) is found in the second row (D-F). Cells labeled with EDC conjugated particles at a higher concentration (50 μg Fe/mL) were analyzed in the final row (G-I). Antibody mediated binding was observed for anti-CD20 SPIO using both conjugation strategies; however, cell labeling with CuAAC nanoparticles was achieved with a significantly lower concentration of nanoparticles. Solid lines refer to unlabeled B cells, dashed to cells incubated with NP of type indicated by row and column.
Overall, the data presented here provide strong evidence that CuAAC reactions can be used for highly efficient and effective labeling of nanoparticles with antibodies, without any noticeable loss in antibody functionality. Fluorescent micrographs of click conjugated SPIO particles incubated with B cells are also shown (Figure 6.6). Labeling of the cells was found to be specific, as IgG-SPIO conjugates do not bind to the cells, nor do anti-CD20-SPIO when inhibited with excess free antibody.

It should be noted that the incubation of anti-CD20-SPIO with the B cells, and especially in the presence of free anti-CD20, appeared to have a negative effect on the health of the cells, if only inferred from cell morphology. No cytotoxicological studies were conducted using the constructs created in this work as this targeting agent is itself administered to deplete cells. Logically this could lead to a further functionality study wherein each conjugate, EDC and click, is viability tested for B cells in vitro or in vivo. This related study would be interesting; however, as the mechanism of anti-CD20 mediated cell death is not fully understood, it is beyond the scope of this work.
Cells were incubated for 30 minutes with 50 µg Fe/mL of (A) IgG-SPIO, (B) anti-CD20-SPIO and (C) anti-CD20-SPIO in the presence of excess antibody, and then washed in triplicate. The fluorescently labeled particles are found bind to cells specifically, as competition for ligand curtails cell signal. All images at 40x magnification.
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6.5 - Conclusion

Advanced applications in the areas of diagnostic imaging and drug delivery stand to benefit from antibody- or protein-based targeting of nanoparticle carriers. Preparation of such targeted nanoparticles can however be inefficient and costly. Here, we have shown that the utilization of the CuAAC for the production of antibody-nanoparticle conjugates permits materially efficient and functionally competent targetable probes. In comparison to classic carbodiimide-conjugates, CuAAC adds a valuable alternative to the toolbox of the bioconjugate chemist. This mechanistically simple and highly efficient reaction, which is generally insensitive to reaction conditions, is expected to allow for an expansion in applications for nanoparticle-targeted systems in the clinic.

The products of the click reaction were functionally specific for B cells in vitro, as demonstrated by inhibitory competition with free protein. A preferred targeting ligand for this work is a non-depleting antibody to a receptor that internalizes cargo once bound, and recycles to the surface. This work has implications towards the use of targeted nanoparticle conjugates following the choice of such a suitable targeting vector. This would allow for the intracellular accumulation of the contrast agent. Coupled to a NP for diagnostic or tracking purposes, such an agent would be a powerful means to image the distribution of specific cell types in vivo.
Chapter 6

6.6 - References


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CHAPTER 7. SPIO Enabled Detection of Transient Cervical Nerve Root Injury

7.1 - Overview

Cervical radicular pain is a widespread neurologic condition resulting from the compression of a cervical nerve root. The impact on quality of life and economic cost of persistent states of pain are massive. Current diagnostic imaging approaches fall short in their ability to accurately correlate pain symptoms to evidence of neuropathy.

This chapter focuses on our aim to utilize systemically administered SPIO for enhanced identification of injured nerve roots in a rat model. The chapter begins by detailing the salient epidemiological and economic impact resulting from back and neck pain. An introduction to the anatomic and biomechanical foundations of transient nerve root compression and the resulting axonal degenerative effects are also discussed. A rat model of neuroinflammation following transient nerve compression that has been previously shown to produce sustained states of hypersensitivity is outlined. In order to visualize neuroinflammation in vivo it was necessary to develop a magnetic resonance (MR) imaging protocol suited to the task of utilizing SPIO as contrast. Preliminary experiments were conducted to ensure that a high-field strength small animal MR system, a surface-receive coil and a T2*-weighted gradient echo sequence would enable detection of cell loaded SPIO.
The sequence parameters and animal handling protocol were initially optimized to enable detection of accumulated SPIO. These settings were then utilized for detection of neuroinflammation from a painful transient compression of the C7 nerve root following SPIO administration. Pre- and postcontrast scanning sessions were conducted following a 6 day recovery period from surgery. Imaging at the site of C7 nerve root injury using SPIO resulted in an average postcontrast enhancement of 72.9±31%, measured as the difference in normalized relative signal intensity (nrSI) between ipsilateral (compressed) and contralateral roots. This was significantly greater than the 5.3±12.9% difference in nrSI of injured animals precontrast. Finally, implications of this work regarding future development of imaging in pre-clinical pain models as well as clinical relevance for patient management are discussed.
7.2 - Introduction

7.2.1 - Epidemiology and Impact

Low back pain and neck pain are among the most common physical conditions requiring medical care globally. The healthcare burden for treating spinal pain is considerable, making it the most common physical condition for which a patient elects to see his/her doctor in the United States (1).

The affected portion of the population is large as cervical neck pain has a lifetime prevalence reported to be as high as 71% (2, 3). Further, there is no specific epidemiological demographic; four out of five neck pain diagnoses (81%) were for persons between the ages of 18 and 64 (4). The corresponding economic impact is equally large, with estimates of annual societal costs of $29 billion (5) and work days lost to back pain has been estimated at 186.7 million for 2004 in the US (1).

7.2.2 - Relevant Cervical Anatomy

The central nervous system (CNS) proximal to the cervical spine, consists of 7 vertebrae and 8 pairs of cervical nerve roots (in both rodents and humans). The purpose of the vertebrae are to contain and protect the spinal cord, support the skull and enable diverse head movement. The spinal cord passes through the spinal canal and is surrounded by a protective dural sheath. The spinal cord consist of tracts of white and grey matter. The white is responsible for conveyance of information to and from the brain, while gray matter houses cell bodies and unmyelinated axons. The first two cervical vertebrae have a unique structure in comparison with other components of the
spine, however the lower (C3-C7) vertebrae are similar. The vertebra progress in size from cephalad to caudad with C7, the vertebra prominens, possessing a spinous process larger than the process associated with the first thoracic vertebra (6).

In the schematic of the fifth cervical vertebra (C5; Figure 7.1A), the space through which the C6 nerve roots exit are labeled as the transverse foramen. It should be noted that the nerves of the cervical spine take the name of the pedicle above which they exit (unlike nerves in the thoracic and lumbar spine). The exception is the eighth cervical nerve root, which exits between C7 and T1. The nerve roots of the cervical spine pass laterally at each level to exit from the spinal canal at the same foraminal level as their origin from the spinal cord, Figure 7.1B.

Each level sees six to eight rootlets exit the spinal cord to be joined together and surrounded by the dura to form the nerve root (Figure 7.1C). The dorsal nerve roots are approximately three times as thick as the corresponding ventral roots, except at C1 and C2, because of the greater amount of sensory fibers. The roots of a particular level contain axons whose cell bodies reside in the corresponding dorsal root ganglion (Figure 7.1C). Each dorsal root level enervates a specific area of skin. These often overlapping areas are the dermatomes. A dermatome map for the cervical and first thoracic nerve root levels is shown in Figure 7.1D. Although variability differences can complicate diagnosis (7), pain in a particular dermatome can enable distinction of the compromised nerve root level in an injured patient (8).

The upper quarter of the foramen is filled with areolar tissue and small veins. In addition, small arteries arising from the vertebral arteries and the sinuvertebral nerves traverse the canals (6). The intervertebral foramen is also in close proximity to the
uncovertebral and the apophyseal (facet) joints (anteromedially and posterolaterally, respectively). These features, the somewhat dense vasculature and adjacency of joints, are noted because they are potential causes of canal (neuroforamenal) restriction. There exist a host of causes that produce neck pain including degenerative conditions, strain injury and direct trauma (9, 10). Cervical radicular pain is distinguished as pain derived from insult to the nerve roots (11). The incidence of either a lesion or contact by the uncinate processes, facet joint or pedicles will result in compression of the nerve roots or spinal nerve which may result in this painful injury, as will be discussed in the following section.
Figure 7.1 - Cervical Neural and Vertebral Anatomy.

(A) Description of the cervical vertebral architecture. (B) Schematic of the spinal cord with nerve roots passing laterally through the neuroforamen to enervate the body. (C) Schematic of the rootlets as they branch together to form the roots and subsequently the spinal nerve. (D) Anterior and posterior dermatomes of the cervical spinal nerves and first thoracic level.
7.2.3 - Cervical Radicular Pain

Cervical radicular pain is a neurologic condition characterized by dysfunction of a cervical spinal nerve, the roots of the nerve, or both. It usually presents with pain in the neck and one arm often severe enough to compromise patients' functional abilities (12). Impact on the quality of life of patients exhibiting symptoms can be severe (13). The condition manifested as pain, sensory loss and motor weakness in the affected dermatome (14) and these symptoms can last for as long as months or even years (15).

Chronic neck pain caused by nerve root injury is common as these roots are at particular risk for mechanical insult (see above). Their risk of compression due to the foraminal shape changes that may occur during some vertebral motions is high (16). Cervical radiculopathy may result from herniation of the nucleus pulposus, however this is less frequent than in the lumbar spine (17). Prevalent causes of such trauma leading to persistent pain include rear-end automobile accidents (18) and contact-sports injuries (19-21). Extensive, large sample population studies have been conducted to determine the effect of suspected risk factors on predisposition to cervical radiculopathy. These are thought to include tobacco-use, psychological health, trauma, sex, age and degenerative disc changes (15). However, the condition also can arise in patients devoid of putative risk factors (17).

The most commonly reported injured spinal nerve or nerve root level is that of C7, at the C6-C7 vertebral level. One study revealed that in a population of 561 patients, the frequency of surgically confirmed cervical radiculopathy at C7 alone was 46.3% (17). Injury at this level produces pain distributed on the affected side along the shoulder, posterior of arm and forearm and along the third finger of the hand (22).
7.2.4 Imaging Techniques in Nerve Root Injury

Clinical criteria for diagnosis of cervical radicular pain lacks a universal standard (23). Application of guidelines following a physical examination are used to screen patients' symptoms from life-threatening pathologies (such as spinal tumors or cerebral infections) (24, 25). These tests, which help confirm or eliminate clinical diagnoses, also serve to localize the injured spinal level by analyzing the affected dermatomal area.

Clinical imaging modalities of all types are employed in determination of the cause of severe and chronic neck pain. The first line of diagnostic tests to evaluate suspected radiculopathy are often conventional radiographs. These may be helpful in assessing for abnormalities such as fractures or instability. However, this technique is of limited utility as it lacks soft-tissue distinction and the degenerative hard-tissue changes in geriatric cases further complicate diagnosis (26, 27). While not immune from asymptomatic findings, magnetic resonance (MR) imaging is the modality of choice for cervical radiculopathy (28, 29). This is a result of the excellent soft-tissue contrast achievable through application of different sequence protocols.

Radiological evidence of neural impingement, such as disc collapse and/or protrusion, spondylosis, or foraminal occlusion, is seen in as many as 44% of lower back pain (30) and 68% of neck pain cases (17). However, in most of the remaining cases, no noticeable neuropathology is detectable.

The challenge of diagnosing this clinical syndrome is exacerbated by the high rates of positive radiological findings in asymptomatic subjects (31-33). It has been estimated that the prevalence of cervical spondylosis for men above 75 is approximately
95% (34, 35). Patients who report no pain symptoms are commonly found to have degenerative spinal features, which may include nerve root impingement (12). This further impairs the utility of current clinical imaging approaches as it is difficult enough to determine the presence of injury, let alone distinguish extent or response to treatment over time.

It is also possible that this pain is the product of a transient mechanical injury or presence of chemical factors that are not apparent through conventional diagnostic radiology. Nerve root insult originating from these sources may not result in cervical neural or musculoskeletal deformation that can be detected using conventional radiographic approaches. We have hypothesized that the application of a cellular MR imaging strategy leveraging the accumulation of systemically injected SPIO to generate inflammation specific contrast can be used to identify the site of a pain producing injury.

**7.2.5 - Neuroinflammation**

It is generally accepted that radicular pain results largely from mechanical or chemical insult of the nerve roots (36-38). Trauma to the neural tissues results in both pain and Wallerian degeneration (39-42). In lumbar radiculopathy models of pain, behavioral hypersensitivity is immediate and has been correlated to axon cytoskeletal damage, cytokine production and glial activation (43-46). Contributing to persistent pain is the neuroinflammatory cascade that sees recruitment of macrophage to the injured region to phagocytize myelin debris (40, 47, 48) followed by further production of small molecule pain mediators (45, 49-51).
Sustained neuroinflammation can be induced by even transient mechanical injuries to neural tissues (36, 45) and may explain the lack of correlation between radiologically evident pathology and pain. In a rat model of cervical radiculopathy, transient compression of the C7 cervical dorsal nerve root produced persistent behavioral sensitivity, as measured by mechanical allodynia (sensitivity to an otherwise non-painful mechanical stimulus) (52-54).

Similar to transection and crush models, transient compression produces Wallerian degeneration, along with the hallmarks of neuroinflammation, including increased upregulation and expression of neuropeptides, growth factors, and cytokines, and sustained inflammatory cell activation at the injury site and in the spinal cord (45, 50, 55). Infiltrating macrophage ingest axonal debris at the injury and have been shown to be a principal element in development of pain (48, 56, 57). The inflammatory response associated with pain states incited by transient neural tissue trauma may provide an opportunity for identification and diagnosis of injury in subjects that present no radiological abnormalities.

### 7.2.6 - SPIO Accumulation at Sites of Inflammation

Superparamagnetic iron oxide (SPIO) nanoparticles are a widely used $T_2^*$-weighted MR contrast agent. They enable detection of sites of their accumulation through enhanced proton dephasing (darkening), as they function as strong magnetic inhomogeneities in vivo (58). They have been applied in numerous academic and clinical studies in a wide range of models for the detection of inflammation. Imaging of these sites using SPIO has been previously shown to rely upon the accumulation of the
particles at sites of activated immune cell activity; specifically they are sequestered by monocytes (59-61). Following sufficient accumulation in foreign-body phagocytosing macrophage, SPIO have enabled detection of otherwise radiologically indistinguishable inflammatory processes. These include pathologies such as soft-tissue infection (59, 62, 63), implant rejection (64) and atherosclerosis (60, 65-68).

The agents have also been widely used in several central nervous system disorders for the detection of inflammatory cells. One of the most widely studied applications is the detection of cerebral lesions in multiple sclerosis (69-72). In humans, sites of inflammation following stroke have also been visualized through SPIO contrast (73). Peripheral and central nerve injury models have been investigated as well. The distribution of macrophages into a mechanically crushed sciatic nerve in a rabbit were tracked following SPIO administration (74, 75). SPIO have been used in the cord injury models to enable visualization of either macrophage in vivo or to track ex vivo loaded macrophage or stem cells to the injury (76, 77).

There are currently no reports of in vivo cellular imaging studies investigating the use of SPIO to identify and/or localize the anatomical location of pain resulting from transient neural injury. As indicated above, a painful cervical nerve root compression model has demonstrated that transient compression of sufficient force is able to produce a sustained inflammatory response (36, 45). It is the aim of this chapter to utilize SPIO to exploit the phagocytic activity of macrophage infiltrating the injury site as schematically illustrated in Figure 7.2. Prior to the application of SPIO, it was necessary to determine optimum MR scanning logistical and sequence parameters for cell-accumulated SPIO detection.
It is hypothesized that systemically injected long circulating dextran-coated SPIO will be able to accumulate in phagocytic macrophage at the site of nerve root compression. Transient compression (red clip) of the C7 dorsal nerve root has previously been shown to lead to neuroinflammation and the presence of activated macrophage (purple). SPIO (black) have been widely used to visualize sites of tissue injury through accretion of nanoparticulate contrast within macrophages.
7.3 - MR Protocol Development

The noninvasive high-resolution imaging of living subjects is a primary advantage in utilizing MR imaging for preclinical studies. A major difficulty, and the underlying motivation for the application of contrast agents, is inherently low signal-to-noise (SNR). This is often the reason for long scan duration in the clinic, as an initially long acquisition must be averaged several times to reduce background noise from otherwise useful information. The aim here was to visualize the accumulation of nanoparticulate contrast agents at sites of neuroinflammation, a difficult task from an SNR standpoint. This was due in large part to the complex biomechanical function of the cervical spine.

A precise assemblage system of bone, cartilage, ligaments, tendons, nerves and muscles all exist to enable both a high degree of flexibility and protect the spinal cord from injury. To perform successful neuroinflammatory imaging, the relevant structures need to be defined with adequate resolution. The substantial artifact that can arise from the presence of bone is also a significant concern. Finally, the spinal nerve roots are located at some distance from the back of the neck in the rat. The further the structure of interest is from the receiver coil, the more signal that is lost for image formation. In order to ensure that equipment parameters were properly configured for SPIO detection sequence, high field strength, sequence selection and the use of specialized coils were examined.
A constant feature throughout the history and development of magnetic resonance imaging systems has been implementation of greater field strengths. Industrial production of clinical scanners have progressed from 0.2-0.3 T systems to 1.5 T and now the current standard of 3 T. Higher field strength produces images of better quality as a result of improved signal-to-noise. This is a result of the differing effects on signal and noise with increasing field strength (B), as they have different magnetic field-dependencies (78).

Strength of signal from a voxel is proportional to voxel volume. The SNR is proportional to voxel volume multiplied by the square root of the acquisition time. Attainment of greater SNR, using higher magnetic field strength, thus results in reduced time and means that voxel volumes can be reduced to achieve the same signal as at a lower field strength for the same amount of time (79). The resulting features of higher strength fields are faster scans and higher resolution. Faster scans have the added advantage of reducing artifacts from patient motion, as the likelihood of movement increases with duration. Thus, all of the work in this thesis (excluding contrast agent characterization) was conducted at high field strength, using a 9.4 T small animal imaging system (Varian; Palo Alto, CA).

7.3.2 - Coils

When surface coils are applied it is often the case that separate coils are used to excite the subjects’ protons and to receive signal for detection. These two coils are referred to as the transmitting-volume coil and the receiving-surface coil. Surface coils
function as antennas to achieve greater SNR in MR imaging. The closer proximity to the
tissue of interest to be imaged allows for less signal to be lost. The reduced field of view
also serves to reduce noise from surrounding tissues in the volume excited by the body
coil.

In this work a custom built surface coil was utilized in conjunction with a volume
coil. The volume coil was a transmit-and-receive design built by Insight Neuroimaging
Inc. (now InsightMRI), Worcester, MA. This coil was used solely in the transmit mode.
The dimensions were 7 cm in inner diameter and approximately 9 cm in length. The
surface coil was applied directly to the surface of the rodent proximal to the tissue of
interest. This receive-only coil and was detunable with a linear, single-loop coil with
remote tuning and matching. The dimensions of the surface coil (antenna only) were 2.54
cm in long axis, curved to a 1.5 cm radius.

7.3.3 - Sequence Selection

The usefulness of MR imaging in the clinical and research spheres is greatly
enhanced by its ability to visualize and distinguish many different tissues. This capacity
is derived from the application of radiofrequency pulses and magnetic gradients in order
to provide distinction of tissues and tissue states of interest. The direction, strength and
timing of the pulses and gradients used to acquire an image is referred to as a sequence.
Sequences are essentially exercises in choosing between speed, contrast and SNR in
order to visualize items of interest. This is relevant here as choice of the sequence in
order to emphasize the presence of susceptibility generating contrast agents is often
necessary. Determination of favorable parameters for detecting intracellular SPIO
contrast agents was thus required prior to any attempt to visualize transient nerve root compression.

Initialization of a susceptibility enhancing sequence, and all subsequent imaging was performed on the University of Pennsylvania Department of Radiology small animal 31 cm diameter 9.4 T MR system (Varian, Palo Alto, CA). Initial results, using the volume/surface coil setup detailed above, indicated high SNR at sufficient tissue depth (maximum visualization of approximately 3.5 cm). A gradient echo multi-slice (GEMS) sequence was selected as multiple animals were to be imaged in each group (requiring efficient scan duration) with thin slice selection (0.5 - 1 mm) along with the desire for a susceptibility bias. In other words: fast, selective and T$_2^*$-weighted.

The fast scanning time results from several GEMS features including a non-90° flip angle and absence of a 180° rephasing pulse (as is utilized in spin echo imaging). This angle (α), selected as 20°, is used to decrease the time required to return to equilibrium magnetization. This is the result of the decreased magnitude of the transverse magnetization, as compared to a 90° or hard flip wherein the entire magnetization vector is (at least initially) in the xy-plane. The shorter recovery period allowed for repetition and echo times (TR and TE, respectively), as shown in Figure 7.3. This has the advantage of minimizing acquisition time and limits breathing and other motion artifacts. As long as a non-steady state longitudinal magnetization does not interfere with imaging (a possible interfering side effect of <90° flip angle when the TR < T$_2$) the slice thickness chosen can be as thin as the gradient allows (on the system used the minimum was 0.5 mm).

A final advantage of the GEMS sequence is that T$_2^*$-weighting is present. There is only a single radiofrequency pulse delivered (to flip) and no 180° refocusing pulses (as
there would be in, for example, a spin-echo sequence). Thus, relaxation process from magnetic field inhomogeneities (such as SPIO) are not corrected for. The trade-off of such a feature is that a dephasing gradient needs to be applied prior to the readout gradient. This process, in addition to the lack of rephasing pulses, results in some loss of signal.
The radiofrequency (RF) pulse used in the gradient echo sequence to flip the net magnetization vector into the xy-plane is by some (non-90°) angle $\alpha$. The value used for all imaging was 20°. This limits the time for recovery of longitudinal magnetization allowing shorter TR and TE to be used. No 180° refocusing pulse is applied, which further speeds scanning time and also does not correct for magnetic inhomogeneities. This results in $T_2^*$ rather than $T_2$-weighting. In the absence of a refocusing pulse the echo is created by applying a dephasing gradient during slice selection ($G_{SS}$) before the signal is read during application of the readout gradient ($G_{RO}$). This generates an echo when the the data is acquired by the analog to digital converter (ADC). The phase encoding gradient ($G_{PE}$) is varied in order to fill the k-space. Adapted from the Vnmrj user manual.

Figure 7.3 - Gradient Echo Sequence.
7.3.4 - GEMS Parameter Optimization

The GEMS sequence was selected as a partial flip angle reduces acquisition time and enhances susceptibility artifacts. However, there exists a significant compromise in the application of gradient echo protocols with the TE parameter. The T$_2^*$-weighting produced due to the lack of rephasing pulse means that signal loss is rapid and there is a low SNR. To maintain the advantage of a fast acquisition time, it was undesirable to resort to reliance on extensive averaging to recover lost image quality. In order to determine a sequence parameters that balanced T$_2^*$-w and image quality a mock SPIO-intracellular-accumulation experiment was conducted.

Initial experiments had indicated that sufficient signal could be obtained from the dual-coil setup with TR in the range of 100-200 msec. Longer TR resulted in extensive breathing artifacts (see Figure 7.4F). Very short TR were not feasible because of the thin slice selection requirement; adjacent thin slices interfered with each other with short TR due to presence of remnant magnetization. After qualitative experimentation, a TR of 200 msec was chosen for the following experiments.

As TE optimization was intended to maximize distinction of the presence of SPIO, a simulated in vivo SPIO-cell experiment was devised and analyzed. Specifically, cationic, cross-linked and aminated SPIO (33 nm diameter, R$_2$ of 72 mM$^{-1}$Sec$^{-1}$) at 100 µg Fe/mL were incubated with confluent human embryonic kidney (293T, ATCC) cells for 1 hour. These cells have previously been shown to be excellent candidates for transfection through relatively efficient uptake of nanoparticles during incubation (80, 81). The cells were washed of excess SPIO after the 1 hour incubation, trypsinized and washed twice in PBS. The cell suspension, containing approximately 2.5x10$^6$ cells, was
delivered by injection intramuscularly to the back of the rat neck, at a depth of approximately 1 cm.

The anesthetized animal was placed into a custom-built stereotactic restraint device after induction at 4% isoflurane in oxygen. The surface coil was fixed proximal to the general area of the injected cells. A coaxial delivery and vacuum system was used to maintain the animal at 1 L/min of a reduced (2%) gas mixture. The animal was then placed on a custom-built patient bed and centered in the 7 cm inner diameter volume coil.

Initial scouting scans were acquired in all three directions in order to determine the relative position of the injected SPIO-loaded cells. Sagittal scans (13 slices) were then acquired using the following parameters under the GEMS sequence: TR = 200 msec, np = 256, nv = 256, N = 4, a field of view of 60 mm² and a flip angle of 20°. A span of TE values from the minimum (2.72 msec; bounded by bandwidth constraints) to the maximum (11.25 msec; bounded by TR) were then used for acquisitions. Representative images of the scanning results are found in Figure 7.4A-E.
The determination of an echo time (TE) for the applied gradient echo sequence was necessary. This parameter allows for distinction of the presence of iron oxide nanoparticle labeled cells, while balancing other affects that impact image quality. Images (A-E) are of the same animal with increasing TE and the following parameters; 256x256, 4 averages and TR = 200 msec. The TE was varied from 2.77, 3.88, 5.00, 10.00 and 11.25 msec. Contrast to noise measurements were conducted to determine the optimal TE. Longer TR times, for example 400 msec (as shown in F) resulted in significant artifact from animal respiration.

Figure 7.4 - TE Optimization Using SPIO-Cell Implant.
Quantitative analysis was undertaken to determine the TE value that provided T₂*-weighting with sufficient image integrity. Using ImageJ (NIH; Bethesda, MD) the images were converted to a useable lossless format in the Multi FDF Opener plug-in (Shanrong Zhang, University of Texas Southwestern Medical Center). A constant area (15 pixel diameter) circle ROI was placed on the injected cells, adjacent back muscle and in a noise-field surrounding the animal, as illustrated in Figure 7.5A. The contrast-to-noise ratio (CNR) was computed for each of the acquisitions.

The results indicated that a TE between 3.88 and 10 msec provided the greatest CNR, Figure 7.5B. Longer TE times (Figure 7.4D-E) were qualitatively the most striking as greater time for susceptibility artifact generated hypointensity of the spinal vertebral column as well as the bones of the rat forepaw. It should be noted that T₂*-weighted inhomogeneities are generated by both bone and SPIO contrast. Thus, increasing the effect for one serves to enhance the other. Bone's artifact generating properties are a result of several factors including a lack of water, a sharp interface with soft-tissue and radiofrequency penetration and reflectance. This presents a dilemma to the operator or radiologist as images of anatomical features that may be highlighted with nanoparticles which are close to or bounded by bony structures may suffer from interference and degraded image quality. Gains in contrast enhancement between the SPIO and surrounding tissue is offset by the distortion and artifact generated by the bone.

Lower TE times provided sharper distinction of tissues as there was less signal lost, however the midrange TR applied (200 msec) coupled with a very short TE (for example 2.7 msec) limits the T₂-weighting of the scan. Using a TE of approximately 5-6
msec in practice enabled excellent soft-tissue distinction, acceptable bone susceptibility artifact and sensitive SPIO-cell detection.
Figure 7.5 - Analysis of TE Adjustment.

(A) An illustration of the ROI used to calculate the CNR for determination of acceptable values. Three ROI, of equal area, were placed on each image; SPIO-loaded cells (dotted), a background tissue (solid) and the noise (double-lined) ROI are shown. For each image the placement was nearly identical however small changes in the location of features resulted from animal movement and TE choice from scan to scan. (B) The results of CNR are plotted, as a function of TE. CNR values were found to be maximum between values of 3.88 and 10 msec.
Chapter 7

7.4 - Materials and Methods

7.4.1 - Nanoparticle Synthesis and Characterization

SPIO were prepared using the chemical co-precipitation technique (82). All chemicals and equipment were purchased from Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Briefly, a solution of iron salts, 1.97 g of FeCl\(_3\) and 0.7313 g of FeCl\(_2\) (each in 12.5 mL of dH\(_2\)O) were dissolved in the presence of 25 g dextran-T10 (GE Healthcare; Waukesha, WI) in 50 mL dH\(_2\)O under nitrogen gas. The vessel was cooled to 4° C and 15 mL of ammonium hydroxide was added. This solution was then heated to 90° C for 1 hour, cooled overnight and then purified from aggregates by ultracentrifugation at 20 kRCF. The supernatant containing nanoparticles was diafiltered against greater than 20 volumes of 0.02 M citrate, 0.15 M sodium chloride buffer, using a 100 kDa cutoff membrane (GE Healthcare). Finally, particles were exchanged into pH 7.4 phosphate buffered saline (PBS), 0.2 μm syringe filtered and stored at 4° C.

SPIO were characterized by dynamic light scattering (DLS) to determine hydrodynamic diameter using a Zetasizer Nano-S (Malvern Instruments; Malvern, UK). The transverse relaxivity, R2, of the particles was calculated as the slope of the 1/T\(_2\) against iron concentration. T\(_2\) relaxation times were determined using a Bruker mq60 MR relaxometer (Bruker; Milton, Ontario) operating at 1.41 T (40° C) using a standard Carr-Purcell-Meiboom-Gill mulitecho sequence (τ=1.5 msec and 2 dummy echoes) fitted using a monoexponential decay.
Male Holtzman rats (Harlan Sprague-Dawley; Indianapolis, ID) were housed under USDA- and AAALAC-approved conditions with free access to food and water. All experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and carried out under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (83). At the time of surgery all rats weighed between 275-332 g, Table 7.1.

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**Table 7.1 - Participating MR Imaged Rats.**

Rat subjects used in this study are labeled by their chronological surgery number (by neurosurgeon Christine Weisshaar). The date of surgery (day 0; see Figure 7.6) is given. All compressions were as described; 15 minutes at 10 gmf. The weights reported are immediately prior to surgery.

Surgical procedures were performed under inhalation anesthesia using 4% isoflurane for induction and 2% for maintenance. The transient unilateral nerve root compression injury has been previously described and reliably induces sustained
behavioral sensitivity that lasts for nearly 3 weeks (16, 40, 84, 85). Briefly, a hemilaminectomy and facetectomy were performed at the C6-C7 level of the right side to expose the C7 roots. For painful injury, a microvascular compression clip (World Precision Instruments, Inc.; Sarasota, FL) was used to apply a 10 gmf compression to the dorsal nerve root for 15 minutes (n=6; Figure 7.6A). Identical surgical procedures were carried out for the sham group except that the nerve root was exposed only, without any compression (n=4). The surgery site was sutured and closed with surgical staples. Rats were continuously monitored following surgery.
Figure 7.6 - Experimental Design.

(A) The painful injury is provoked by transient compression of the right-side C7 dorsal nerve root. A haemostatic compression clip (*) is applied proximal to the dorsal root ganglion (DRG), following the disclosure of the nerve root. The ventral ramus (VR) and dorsal ramus (DR) are also indicated. (B) The timeline for the experiment shows that 6 days following surgery precontrast images of the animals are acquired in a 9.4 T MR. SPIO are tail-vein injected following the pre-contrast imaging session and a postcontrast scan is performed 24 hours later.
7.4.3 - MR Imaging

All experiments were performed using a 9.4 T horizontal small animal MR equipped with a 25 G/cm gradient insert (Varian; Palo Alto, CA) at the Small Animal Imaging Facility in the Department of Radiology at the University of Pennsylvania. Imaging was performed using a dual-coil system; a 7 cm diameter volume coil was actively detuned for use with a 2.5 cm loop receive-only surface coil (InsightMRI; Worcester, MA).

Rats were anesthetized prior to imaging, as described above, and placed supine into a custom-built stereotactic restraint device that held the surface coil to the cervical spine. A coaxial breathing apparatus delivered anesthetic throughout the scan and removed CO₂ and excess isoflurane. Temperature and electrocardiography probes were used to monitor the rats in the 37°C environment supplied by an air heating system (SA Instruments, Inc.; Stony Brook, NY).

Precontrast image sets were acquired at day 6 following surgery (Figure 7.6B). After the precontrast imaging session, 10 mg Fe/kg (approximately 300 μL) was delivered systemically by tail vein injection. Postcontrast image sets were acquired 24 h later, on day 7 following surgery (Figure 7.6B). Identical imaging procedures were used for both of the pre- and postcontrast imaging sessions. In vivo MR microscopy of the C6 and C7 spinal cord and nerve roots was performed using the VnmrJ software (Varian). The MR imaging protocol consisted of several scout sequences to locate the C6 and C7 nerve roots. This was followed by a T₂*-weighted gradient echo multislice sequence (TR 200 msec, TE 5.5 msec, 20° flip angle) in the axial plane. Greater T₂*-weighting, while desired, was precluded due to proximity (and enclosure) of interrogated anatomy to bone.
Fifteen interleaved slices were typically acquired, with a thickness of 0.5 mm, NEX = 8, nv = 256, np = 256 and a field of view of 5 cm x 5 cm.

7.4.4 - Image and Statistical Analysis

Image sets were analyzed using ImageJ (NIH; Bethesda, MD), following their conversion using the Multi FDF Opener plug-in (Shanrong Zhang, University of Texas Southwestern Medical Center). User defined ROI of equal area were set for the ipsilateral and contralateral (control) C6 and C7 nerve roots, as well as two background (paraspinal muscle) locations, under radiologist-blind (pre-, postcontrast, injured or sham) conditions.

On a slice-specific basis, the relative signal intensity, rSI, was calculated by division of the mean intensity of the ROI at each nerve root by that of the paraspinal muscle. For each subject, this value was then normalized to the rSI maxima of the C6 nerve root to give the normalized rSI (nrSI) for ipsilateral and contralateral roots. Quantitative comparison of the pre- and postcontrast C7 nerve roots, as well as between injured and sham groups, was made possible by then computing the difference of the normalized rSI peak and immediately adjacent slices'. MR findings, the presence or absence of enhancement, were considered significant if $p < 0.01$. A two-tailed Mann-Whitney test was used.
Tissue was harvested from all rats immediately following the final imaging session on day 7 (Figure 7.6B). Transcardiac perfusion was performed using PBS and then a 4% paraformaldehyde-in-PBS mixture; the cervical spinal cord was exposed and the C7 level was bisected to enable removal of the nerve roots and spinal cord en bloc on each of the ipsilateral and contralateral sides separately. Tissue samples were embedded in paraffin to enable axial sectioning (10 μm thick). Nanoparticles were localized in the tissue by Prussian blue staining for iron oxide using a wash of a 1:1 mixture of 2% potassium ferrocyanide and 2% HCl for 20 minutes. Sections were rinsed in dH2O, dehydrated, cleared in Citrisolv and then cover slipped using Permount. Serial sections were also immunostained to co-localize macrophages with SPIO; activated macrophages were stained using an antibody against the CD68 receptor (ED-1, Serotec; Oxford, UK). A horse anti-mouse secondary antibody (Vector; Burlingame, CA) was used and developed using the ABC kit (Vector). Sections were dehydrated, cleared and then mounted.
7.5 - Results and Discussion

Precontrast images were acquired after 6 days of recovery from surgery. Dextran-coated SPIO, with a 29 nm hydrodynamic diameter and R₂ of 104 mM⁻¹ sec⁻¹, were then injected at a dose of 10 mg Fe/kg. The following day imaging was repeated. The cervical nerve roots at the C6 and C7 NR levels were visualized with 0.5 mm thin axial slices in series using a T₂*-w multi-slice gradient echo sequence.

Representative axial acquisitions from each group, pre- and postcontrast, are shown in Figure 7.7. Scans of the rat cervical spine show the spinal cord at the center surrounded by vertebral bone enclosed in muscle. Careful axial slice selection afforded visualization of both the ipsilateral and contralateral roots in the field of view, allowing for an inherent control (contralateral root) to be included throughout imaging. Bilateral roots can be readily identified in the precontrast images for both injured and sham groups. There is a clear loss of signal in the ipsilateral (compressed; Figure 7.7D) root in the postcontrast imaging session.
Representative MR acquisitions of spinal cord and nerve root level C7 (C6 vertebral level). Use of a dual coil arrangement afforded high resolution imaging at tissue depths of several centimeters. The spinal cord (SC), vertebrae (V; especially the anterior tubercle), disc (IVD), nerve roots (ipsilateral and contralateral; Ip, Co respectively) and wind pipe are clearly visible for each subject. Soft tissue filling of the void created upon hemilaminectomy bone removal to expose the nerve roots is present to differing degrees (indicated below symbol □ in A). The pre- and postcontrast images for the sham animal, A and B respectively, show little signal difference in nerve roots on the exposed side. In contrast, the pre- and postcontrast acquisitions for the injured subject, C and D, differ at the transiently loaded NR. Indicated by the arrow, a signal void is present at the NR following administration and accumulation of SPIO.
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The placement of ROI used for quantitative analysis is given on a representative acquisition in Figure 7.8A. Here, the spinal cord with roots passing through the surrounding vertebrae at C7 is shown. The spinal level of interest was localized using the landmarks of soft-tissue injury and anterior tubercles. ROIs were drawn for each dorsal root as well as at sites in the surrounding back muscle. If no nerve root was visible, the ROI was placed in the linearly inferred position between successive roots.

The flexibility of the rat neck in multiple directions meant that axial plane alignment of both ipsilateral and contralateral nerve roots was not always feasible, despite the use of a stereotactic restraint device. Therefore, to ensure that the signal loss at the injured root was a result of SPIO accumulation rather than from imaging artifacts, such as partial volume effects, a series of sequential slices were acquired for each rat to include nerve roots through the C6/C7 levels. A representative series of such acquisitions is shown in Figure 7.8B-F. These five adjacent slices at C6 from a C7-compressed rat show that there is no evidence of signal loss in the ipsilateral root at one level above the injury.
Figure 7.8 - Nerve Root Injury Visualization.

(A) A representative axial image is presented, overlaid with ROI for the ipsilateral (R_i) and contralateral (R_c) roots as well as background paraspinal muscle (B_1 and B_2). The ROI are have been slightly enlarged for clarification. Analyses investigated relative signal through a series of sequential acquisitions to avoid artifactual errors, such as partial volume effects, from distorting the results. An example of 5 consecutive 0.5 mm slices through the C5 vertebral level (C6 nerve roots) are shown raustral to caudal in B - F.
The normalized relative signal intensity (nrSI) computed for each imaging slice in the rostral-to-caudal direction of an animal are shown in Figure 7.9. The periodic maxima reflect the appearance of the nerve roots, while the minima reflect the presence of bone. Qualitatively, it is evident that there is little discrepancy between the ipsilateral and contralateral nrSI in the precontrast series. An example of slight offset of roots, as described above, is seen in Figure 7.9B; here, the bilateral C6 roots are in different imaging planes. Decrease of the nrSI at C7 in the postcontrast injured rat, resulting from SPIO accumulation, is substantial (Figure 7.9D).
Figure 7.9 - Signal Ratio Through Roots.

Raustral-to-caudal NR signal (relative to paraspinal muscle) for a representative sham; precontrast (A) and postcontrast (B), and an injured animal; precontrast (C) and postcontrast (D). Values of relative signal intensity were normalized to C6 maxima. In an evaluation of the nerve root rSI, periodic increases of signal were found to correlate with the appearance of roots. The signal minima correspond to bone and intervertebral tissue. Sham and precontrast injured subjects did not demonstrate discrepancy in normalized signal ratio intensity between C6 and C7 nerve roots for the ipsilateral (dashed) and contralateral (solid) sides. When investigating postcontrast injured animals a large difference in the C7 nerve root signal intensity ratio was observed, (NR = nerve root)
To quantitatively compare the sham and injured groups, pre- and postcontrast, the percentage difference between the contralateral and ipsilateral nrSI was calculated (double ended arrow, Figure 7.9D). These values are plotted as percent change in Figure 7.10. SPIO accumulation over 24 hours led to local hypointensity and a difference in nrSI between the roots in the injured case of 72.9±31%. No significant change in precontrast images of nrSI was detected between ipsilateral and contralateral nerve roots in the injured (5.35±12.9) or sham (-10.8±5.4) animals. This result essentially recapitulates the deficit in clinical MR imaging of radiculopathy; there is no visible difference between injured and healthy nerve roots (without contrast). Further, the sham group showed no significant nerve root enhancement (2.8±14.1%) 24 hours after the SPIO injection.
Figure 7.10 - SPIO Enhanced Detection of Nerve Root Compression.

The quantification of normalized relative signal intensity (nrSI) differences between the ipsilateral and contralateral nerve roots is shown. Precontrast subjects (filled symbols) and postcontrast (open) normalized differences are plotted along with the mean of each group (bold line) and the standard error for each group. Significant enhancement (p < 0.01) between the (**) pre- and postcontrast nerve roots in the injured group as well as between the (*) sham and injured post-contrast roots was found.
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Histological evaluation of the nerve roots and spinal cord was performed to identify the presence or absence of activated macrophages and iron oxide particles. Representative micrographs of dorsal roots and spinal cord are shown in Figure 7.11. The detection of SPIO and macrophage by immunostaining was observed in the injured C7 root (Figure 7.11E-F). Very few inflammatory cells and little accumulated iron oxide was observed at the ipsilateral C6 root above the nerve root injury (Figure 7.11G-H).
Staining of dorsal nerve roots was performed for both macrophage and iron following the final imaging time point. Representative ipsilateral (Ipsi) C7 roots of sham animals were stained for macrophage and iron oxide (A and B, respectively). Neither immune cells, nor SPIO were detected. This was also the case for the C7 nerve roots on the contralateral (Con) side of injured animal (C and D). Activated macrophage staining (E) and corresponding iron (F) was evident on the transiently injured nerve root (ipsilateral side of C7). Finally, limited inflammatory cell infiltration and corresponding iron oxide was found in the C6 ipsilateral root of the injured subjects (G and H). The scale bar in H (equivalent to 100 μm) applies for (A through H). (I) Lower magnification section showing dorsal root ganglion (DRG), dorsal and ventral nerve roots (DNR and VNR, respectively), the dura matter (DM) and one half of the spinal cord (SC). Box denotes approximate area of magnification for (A-H). Scale bar in (I) is also 100 μm.
Generally, the iron oxide was co-localized with macrophages at the injury site; however, the density of macrophage and extensive Prussian blue staining of iron oxide made 1:1 co-localization difficult. A 1:1 localization of iron to macrophage was observed further along the NR, at less dense sites of macrophage infiltration (Figure 7.12A-B). Inflammatory cells and accumulated SPIO were absent in the C7 roots of the sham and the contralateral root of the injured animals. Furthermore, no Prussian blue staining was observed in the injured roots in the absence of SPIO, Figure 7.12C-D.
Figure 7.12 – Macrophage and Iron Oxide Specificity.

(A) Macrophage found at the site of a nerve root compression at day 7 after insult by anti-CD68 immunohistochemistry. (B) These macrophage co-localize with the presence of iron oxide as revealed by Prussian blue stain development in the less macrophage dense regions further distal from the injury site along the nerve root (towards the dorsal root ganglion). Histological investigation of the nerve root reveals that for (C) macrophage, again at 7 days after the injury, there is (D) no non-specific iron oxide accumulation in the absence of a SPIO administration.
7.6 - Conclusion

MR imaging of the cervical spine provides high-resolution visualization of the spinal cord, nerve root and skeletal architecture. However, the difficulty in using this information to identify the anatomic origin responsible for cervical radiculopathy or to correlate pathology with pain symptoms is a significant challenge in clinical patient management (12). Noninvasive markers for the detection of neuroinflammation have significant potential to impact clinical practice through detection and monitoring of therapy for cervical pain.

In animal pain model studies which involve the ligation (44), compression (86) or chemical irritation (46) of neural tissue, hypersensitivity of the affected enervated area is assessed through behavioral responses to mechanical and/or temperature stimuli. In the painful injury model investigated here, compression of the C7 nerve root induces sustained behavioral sensitivity that radiates to the forepaw, mimicking the clinical presentation of radicular persistent pain (57). This same mechanical loading of the nerve root also leads to macrophage infiltration at the root and release of inflammatory cytokines, further contributing to persistent behavioral hypersensitivity (50). In our study, we used SPIO contrast for the in vivo cellular detection of neuroinflammation (53).

It was found that one day following the administration of SPIO, hypointense signal was observed at the injured site on a T2*-weighted image. Inflammation, signified by either hyper- or hypointensity was not detectable at injured roots prior to contrast enhancement (Figure 7.7A-B). Further, non-injured roots exhibited no significant change in signal ratio following contrast delivery. Confirmation that the decrease in signal at the injured site was due to the specific presence of SPIO was accomplished by co-localizing
the iron from the particles to the activated macrophage surface marker CD68 (Figures 7.11-12).

Comparison of nrSI between the ipsilateral and contralateral roots of the injured group revealed a significant change (72.9±31%) in the injured root after SPIO accumulation. No significant percentage difference of nrSI was determined outside of the injured postcontrast group. Small quantities of iron oxide and macrophage were detected by histology at the ipsilateral C6 root of the rats in which the C7 root was compressed; however, this extent of SPIO accumulation was below the MR detection limit. This provides motivation for use of this technique as being specific for detecting the site of injury, providing the potential for adoption as a means to guide surgical interventions for treatment of radiculopathy patients. However, it should be noted that future use of the nrSI metric, which is dependent on C6 root signal intensity, requires that the injury be localized to a single level. This problem could potentially be alleviated through the imaging of additional nerve roots.

In the present study, MR imaging was limited to only two vertebrae for each rat due to the flexibility and depth of the cervical spine, which is situated further from the back than the thoracic or lumbar spine. The surface coil was positioned with the C6 and C7 roots in the field of view, in order to evaluate both a non-injured and potentially-injured level at the same time.

Previous work using SPIO enhanced MR imaging of nerve injury reported hyperintensity at the injury site following compression, followed by darkening upon contrast administration (74). The hyperintense signal feature was not observed here and may have been masked by the bone surrounding the root or may only be present with of
the production of edema which occurs for more severe insults, such as used in that model. A further difference between the two studies was that contrast enhancement was only possible if SPIO were administered 1 day following injury, while the imaging studies here were not performed until 6 days after the initial injury. Earlier investigations of the model employed in this study have revealed macrophage accretion continues for at least 7 days following transient injury (57).

While the work presented here needs to be validated at clinically relevant scanning strengths, it should be noted that many of the anatomic challenges and limitations presented by the rat are not present to the same extent in humans. Cervical vertebrae are well visualized and aligned in the supine position (87). Furthermore, the much larger diameter of the roots in the human (approximately 0.5 cm) would enable greater distinction of bony versus neural tissues and identification of SPIO accumulation.

The setting for which the neuroinflammation detected using SPIO was pre-clinical. However this protocol may provide a basis for the clinical radiologist to correlate cervical nerve root injury to pain. These data indicate that the source of painful neuropathy can be identified 1 day following administration of SPIO and can enable diagnosis of the source of radiculopathy in patients that do not have obvious pathologies. This has been accomplished here using an analogue of commercially available and clinically approved agents (for example the 20-40 nm diameter Ferumoxtran (88)). MR cellular imaging of the site of injury might also allow for personalized, quantitative and real-time monitoring of anti-inflammatory treatment strategies aimed at alleviating radicular pain.
7.7 - References


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CHAPTER 8. Summary and Future Directions

8.1 - Introduction

Use of magnetic resonance (MR) for the tracking of immune relevant cells and delineation of their activity is a challenging engineering task. The potential impact that such techniques possess for enhancing the clinical diagnostic ability of MR and for the understanding of biological phenomena is a powerful impetus for continued advancements in the field. In this dissertation, an adaptable, derivitizable iron oxide nanoparticle platform was developed and applied in several cellular and animal systems.

Under a variety of physical, chemical and electromagnetic examinations the manufactured superparamagnetic iron oxide (SPIO) were well characterized. Several nanoparticle formulations were applied in the determination of key SPIO parameters that affect non-phagocytic cell uptake. Labeling of B cells was accomplished ex vivo. SPIO were used, in conjunction with a near infrared dye to track implanted B cells in vivo with and without the application of B cell depleting antibody. Particles were also utilized to visualize neurinflammation in a rodent cervical persistent pain model. Finally the SPIO were derivatized to accomplish highly efficient, functional, antibody labeling to facilitate in vivo targeting of B cells. The major findings of the work are catalogued in this chapter.
This is followed by a discussion of the likely implications of this work, suggestions for future work and a concise conclusion of results.
8.2 - Significance of Findings

Control over the starting synthesis parameters used in the co-precipitation of the iron salts in the presence of complexing agent was required to produce SPIO of desirable characteristics. Parameters of essential importance were found to include; inert atmospheric conditions, the relative ratio of iron-to-dextran, the rate of addition of reductant and rigid purification practices. The multi-component core (and resultant magnetic properties) and overall diameter of the particles could be modified by altering components of the reaction listed above.

Transmission electron microscopy, dynamic light scattering, viscometry, magnetic relaxation tests and a variety of surface charge probing methods were performed. The manufactured particles possess a non-ideal crystal lattice core of iron oxide likely in a mixed form of maghemite and magnetite, coated in a shell of dextran. SPIO ranging from approximately 20 nm through to 150 nm were synthesized. The surface layer of dextran was altered post-synthesis first through cross-linking the individually FeOH-complexed dextran macromolecules and functionalized with charged residues. Finally, these surface expressed groups were exploited to 1) directly conjugate fluorophores and proteins and 2) as points of conjugation for advanced ligation chemistries, such as the copper catalyzed azide alkyne cycloaddition.

Demonstrated in this work was the ability to functionalize particles with a range of surface properties (with respect to surface charge and expressed groups), physical characteristics (overall diameter, inner core components and magnetic properties) and conjugated moieties. These include but are not limited to fluorophores such as fluorescein isothiocyante, Cy5.5 and Alexa 680; amine reactive and characterizing molecules such
as N-Succinimidyl 3-(2-pyridyldithio)-propionate; click reactive molecules such as amine-reactive propargyl and azide conjugated PEG cross-linkers; and proteins and peptides such as the polyamine protamine and human B cell targeting anti-CD20.

We examined the T<sub>2</sub>-weighted contrast that resulted from by loading non-phagocytic cells with SPIO. The SPIO properties were varied, according to surface charge, hydrodynamic diameter, concentration and time of incubation in order to determine the relevant factors for SPIO labeling of immortalized human T cells. The procedure was verified for primary murine B cells. These cells were then labeled by both SPIO and near infrared (NIR) dye for the purpose of monitoring B cell distribution in vivo in a mouse. The homing of cells to the spleen was verified by both MR and optical methods. The distribution following administration of a B cell depleting antibody was also monitored. These results indicated that SPIO may interfere with the biological process of antibody mediated B-lymphocyte cytotoxicity.

Finally, SPIO were utilized for the identification of the site of neurological insult in a transient compression model. It has previously been observed that transient compression of the rat C7 dorsal nerve root resulted in hypersensitivity of the affected dermatome (1-3). This has been correlated to the infiltration of activated macrophage into the neural tissue (4-6). The presence of these macrophage, was detected through the active uptake of long circulating (non-aminated) SPIO. The degree of contrast enhancement resulting from SPIO administration was determined semi-quantitatively.
8.3 - Future Directions

From both a materials engineering and biomedical study design standpoint, several avenues are available for extending the work completed in this dissertation. Areas of advancement include the refinement of the nanoparticle architecture itself, in vivo targeting of immune system relevant cells and further study of SPIO application to neuropathy.

8.3.1 - Refinement of SPIO

Much of the work in this thesis demonstrated control over the production and physicochemical properties of SPIO for biomedical applications. Advances in materials engineering, however, mean that SPIO with augmented beneficial properties can be constructed. Extending out of the tight control of heterogeneous layered nanoparticles such as type II quantum dots (7), investigation and manipulation of the core-shell relationship has become a major area of research in its own right for magnetic nanoparticles. Two materials in particular would be relevant to coating of iron oxide nanoparticles, gold (8) and silica (9). While no longer a novel prescription, coating the iron with a thin layer of either silica or gold is hypothesized to allow for more robust, and eased, functionalization as the gold-sulfhydryl and silica-binding conjugation chemistries have considerable literature (10, 11).

Advantages of such an approach include better core crystallinity resulting in greater magnetic moment for the particles. This, coupled with the greater functionality, are significant assets when tracking particles or attempting to bind targets in the body.
The disadvantage of such strategies is that synthesis routes are undoubtedly more complex than the iron salt co-precipitation method. If significant work can be completed towards good manufacturing processes of core-shell magnetic nanoparticles, better signal-to-noise, lower dosage and more effective targeting vehicles may be developed.

8.3.2 - In vivo targeting of B cells

The ex vivo loading of SPIO resulted in an apparent inability to detect antibody mediated depletion of B cells in the spleen. This reveals the possibility that an in vivo targeting approach may be better suited for MR detection of B-lymphocyte population migration or demise. The preliminary work towards this strategy has been accomplished. Probes consisting of B cell targeting antibody-SPIO conjugates have been manufactured. This process was determined to make highly efficient use of starting protein. Further, the functionality of the antibody was not degraded. Antibody-SPIO conjugates labeled B cells in vitro with greater avidity than particles developed using the conventional covalent amide linking approach.

The determination of B cell distribution in vivo using antibody-SPIO targeting conjugates is theoretically possible. No study to date has utilized SPIO to specifically label a cell population as populous as B-lymphocytes. However, selection of a targeting ligand (antibody or otherwise) that demonstrates high specificity for B cells with likewise low non-specific adherence to other tissues should allow for identification of sites of B cell concentration. The ability to repeatedly administer a targeted imaging agent should provide a means to identify the change in B cell distribution over the course of a depletion therapy. Furthermore, if the probe is itself benign and well tolerated, it is
conceivable that this SPIO contrast agent could be applied for the study of B cell distribution and response in other autoimmune disease and therapy models.

In a different direction, an interesting observation regarding spleen signal intensity in T₂*-weighted imaging was made. It was noted that the spleens of mice subjected to the immunotherapy protocol, but devoid of nanoparticle MR contrast agent, darkened. This hypointensity was slight and developed over time, however it provides an noteworthy, hitherto unremarked-upon, example of MR imaging of B cell depletion therapy. Any clinically relevant conclusions are certainly premature at this juncture. However if it can be correlated to other therapeutic response markers this phenomenon may provide a non-invasive, agent free, means to determine treatment efficacy.

8.3.3 - Advanced Imaging of Painful Neuropathy

There are several interesting areas in which our work on the in vivo imaging of transient nerve root injury in the rat could be continued. The goal of this work was to address a current deficit in the ability to identify nerve root injury causing radiculopathy in the absence of permanent and gross anatomical changes, such as nucleus pulposa or vertebral degeneration. This preclinical study could be a basis from which to conduct either larger animal (dog or non-human primate) or even human trials. While certainly a leap, human trials may not be beyond the scope of this work in the intermediate future. This assertion is based on the fact that the administered SPIO agents have clinically analogues with similar physical, chemical and magnetic properties that likely would experience comparable accumulation at the site of a pain producing neuropathy (12).
From a cell migration standpoint, it may be possible to repeatedly image the injury in the rat, from insult through to full regeneration of the nerve. This would enable study of the entire process from initial injury to neuroinflammation to an amelioration of that condition. The study model employed for the detection of pain producing neuroinflammation was controlled with respect to the load applied (sufficient to provoke persistent hypersensitivity) and on the days that imaging was carried out. The dates that the imaging was conducted was limited by several factors; namely, the wound closing materials were metallic and it was required that they be removed prior to MR imaging. However, if an alternative could be found and approved, it would be of interest to non-invasively determine, perhaps through repeated SPIO administration, the temporal infiltration of macrophage at early time points following insult.

Imaging approaches also provide a means to perhaps image the severity of injury, which is dependent on several factors such as magnitude of insult and pharmacological management. In the current study, the load and duration of compression were fixed at 10 gmf and 15 minutes respectively. However, it has been previously shown that behavioral outcomes and spinal cellular responses depend on the magnitude of nerve root deformation (13-15). The application of SPIO as an in vivo diagnostic for the gradation of neuropathy could be of significant clinical relevance. In a related capacity, the application of non-invasive imaging techniques could be of tremendous pre- and clinical application to the monitoring of therapeutic efficacy. It has been shown that pharmacological treatment parameters have a significant behavioral and cellular impact in rat pain models. SPIO administration may allow for both imaging of the dynamics of reduction in inflammation following treatment, comparison of therapies and possibly
from a clinical standpoint evaluation of best practices for management of cervical radiculopathy.

Finally, it may be possible to administer magnetopharmaceuticals beyond the unmodified SPIO used in the CNS injury detection studies performed here. Positive contrast agents, such as Very-small SPIO (VSPIO) and Gadofluorine M (an amphiphilic macrocyclic gadolinium complex of relatively low molecular weight) have recently been used to visualize and grade severity of CNS injury in rat encephylitis models (16). Likewise, we have recently developed a nanoparticle formulation that provided excellent tumor signal increase in $T_1$-weighted imaging. This gadolinium-conjugated dendritic nanocluster system has the potential to undergo inflammatory site accumulation through macrophage uptake similar to that observed for SPIO administered in the transient nerve root injury study (17).
8.4 - Conclusion

The foremost goal of this thesis was to observe the presence and distribution of immune relevant cells in vivo. We have demonstrated development of a functional nanoparticle contrast agent for use in magnetic resonance imaging. It was determined that agent properties could be controlled either during the synthesis or subsequently through modification of the surface properties and functionalities of the coating material.

Investigations of the interaction between cellular systems and the produced SPIO revealed that size, concentration and surface charge were critical towards labeling of non-phagocytic cells. An optimal labeling configuration of particle was determined from these studies. In a surprising result, given the widespread use of 20 - 50 nm SPIO, and recent focus on larger micron-sized SPIO, our results indicated that a highly cationic particle of approximately 100 nm standard-SPIO (SSPIO) enabled maximum contrast. The $T_2$-weighted contrast enhancement as measured by a benchtop relaxometer operating near clinical field strength for the optimal particle labeling configuration was approximately an order of magnitude greater than the 33.4 nm ultrasmall-SPIO. Contrast was nearly two orders of magnitude greater than conventional micron-sized SPIO.

To enable lymphocyte tracking in vivo we progressed from an in vitro immortalized cell line to labeling of primary B cells for in vivo administration. In order to avoid detrimental biological effects the less charged smaller particles of 53.5 nm diameter were used. These SPIO and a cell surface membrane labeling near infrared dye enabled MR and optical monitoring of administered B cells in vivo. The primary goal of using these agents to determine B cell distribution in vivo was successfully accomplished. The presence of MR contrast agent in the spleen was confirmed by $T_2^*$-
weighted imaging using a dual surface and volume coil imaging configuration. The detection of hypointensity of the spleen was specific for SPIO loaded B cells. A ratio of splenic signal intensity over background (paraspinal muscle) signal was used to quantify the contrast enhancement. No significant change in signal was determined for controls. The homing of B cells was corroborated by fluorescent signal detection in the spleen from the near infrared dye. Contrast decreased from both modalities over time, as the particles and dye were apparently cleared.

The determination of a change in B cell distribution following administration of anti-CD79 B cell depleting antibody was evident only using the optical approach. Evidently, while the presence of SPIO accumulated in the B cells using the ex vivo loading approach enabled in vivo detection, it also prevented B cell depletion. No significant signal ratio change was detected between the SPIO B cell groups with or without depleting antibody.

We have applied SPIO in a rat pain model and demonstrated cellular visualization of an otherwise occult painful neural injury. The injured nerve roots were readily identified 24 hours following the injection of the contrast. Ratiometric assessment of nerve-to-background tissue specified a significant difference in the normalized signal intensity ratio (nrSI) following administration of SPIO. Imaging at the site of nerve root injury using SPIO resulted in an average postcontrast enhancement of $72.9 \pm 31\%$, measured as the difference in nrSI between ipsilateral and contralateral roots. This was significantly greater than the $5.3 \pm 12.9\%$ difference in nrSI of injured animals prior to SPIO administration.
Future studies will seek to utilize SPIO enhanced MR imaging in order to monitor treatment efficacy and study the 'root' causes of radicular pain. The research reported herein is preclinical, however the protocols established may provide a basis for the clinical radiologist to correlate cervical nerve root injury to pain. These data indicate that the source of painful neuropathy can be identified and may provide the means to diagnose the source of radiculopathy in patients that do not have obvious pathologies. MR cellular imaging of the site of injury might also allow for personalized, quantitative and real-time monitoring of anti-inflammatory treatment strategies aimed at alleviating radicular pain.
8.5 - References


APPENDIX A. T₁ Contrast Agents

A.1 - Gadolinium(III)

The most widely used magnetic resonance (MR) contrast agent (CA) is the paramagnetic lanthanide ion gadolinium (III), referred to herein as Gd. Other paramagnetic ions that perform less well as CA, but are still of interest primarily at the bench-top, are magnesium(II), copper(II) and iron(III) for the same reasons described below. Paramagnetic relaxation of surrounding water protons occurs as the dipole-dipole interaction between the spins of the ion's unpaired electrons and the dipole moments of the surrounding ¹H. In its unfilled d and f shells; the Gd has 7 unpaired electrons, the greatest of any stable ion (1). The resulting effect is a shorter T₁; the nuclei regain their longitudinal magnetization faster and are thus brighter, or hyperintense, on all conventional T₁-w images.

Contrast agents’ ability to relax surrounding protons affects both T₁ and T₂ processes. Gadolinium(III) is a T₁-w contrast agent as the R₂/R₁ ratio of approximately 1-2 means the signal change for T₁-w imaging is much greater than it would be for T₂-w imaging. For comparison, common iron oxide T₂-w contrast agents, discussed in detail throughout this dissertation, often effect an R₂/R₁ of 10 (2).
Appendix A

The enhanced spin-lattice relaxation mechanism of paramagnetic ions has been described in the Solomon-Bloembergen theory (3). Here, observed $T_1$ is due to a combination of the intrinsic $T_1$ of the diamagnetic sample (the protons) and the $T_1$ caused by the presence of the Gd. This latter $T_1$ is composed of two terms, the inner and outer sphere effects. The inner sphere describes the relaxation of the $^1$H directly bound to (hydrating) the paramagnetic ion, and the outer sphere describes the interactions between the $^1$H diffusing as water closely by the Gd (4). The outer sphere effects are difficult to control given that they depend on tissue properties.

Numerous parameters affect the relaxivity of Gd-based MR CA, as demonstrated in Equation A.1, the description of the inner sphere term. For a fixed radiofrequency (RF), $f$, the $R_1$ is a function of the inner sphere water coordination number, $q$ (the number of bound water molecules per Gd), the effective magnetic moment, $\mu_{\text{eff}}$, the internuclear distance of the water proton, $r$, and the correlation time, $T_c$.

Equation A.1

$$f_{R_1} = \frac{q \cdot \mu_{\text{eff}}^2 T_c}{r^6}$$

The correlation time can be represented through the rotational correlation time ($T_r$), electron spin relaxation time ($T_{1e}$) and the inner sphere exchange correlation time ($T_m$), as shown in Equation 1.9.

Equation A.2

$$\frac{1}{T_c} = \frac{1}{T_r} + \frac{1}{T_{1e}} + \frac{1}{T_m}$$

A schematic illustration of these parameters, in a physical sense, is given in Figure A.2.
Critical parameters affecting Gd relaxivity. One inner sphere molecule of water (within the radius depicted by the dashed line) is depicted interacting with the Gd ion. The proton exchange rate is depicted by the bold double arrows. The rotational correlation time of the molecule is shown by the arrow. Adapted by Thorek from (5) and (4).
A.2 - Gd-Chelates and Toxicity

A major issue for use of Gd is that of safety; lanthanide ions are extremely toxic. They must be chelated for biological use to prevent uptake (as well as to provide solubility). Retention of Gd has been shown to cause nephrogenic systemic fibrosis (NSF) in patients with renal conditions (6). NSF is found to cause renal failure, organ complications, skin induration and ultimately death. Confirmation of the diagnosis is primarily made through skin biopsy revealing presence of gadolinium long after administration (7).

Gd-chelating compounds must have very high stability ($\log K_{Gd} \approx 20$) so that the entire agent may be cleared prior to release or replacement of the ion by a metal found in the body (8). Chelation does not abrogate all health risk issues, as transmetallation may still occur to free the ion (9). Clinically relevant chelators can be classed into three primary groups that describe their structural chelation of the Gd as well as their stability, as shown in Figure A.2. In order of most stable (least likely to release Gd) to least stable (weakest chelator), there are: cyclical, ionic linear and non-ionic linear.

While required to prevent cellular uptake of the ion, chelates' osmolality following injection is an issue. Magnevist, for example, is highly hypertonic (Bayer HealthCare Pharmaceuticals, Inc. product information). The agent has an osmolality 6.9 times that of plasma (1973 mOsmol/kg versus 285 mOsmol/kg). Table A.1 contains a list of pharmacological parameters for four of the six U.S. Food and Drug Administration (FDA) approved gadolinium(III) agents.
Figure A.2 - Clinical Gadolinium(III)-Chelate Contrast Agents.

Structure, formula description and trade name of commonly used gadolinium(III) chelators. Dotarem, Gadovist and ProHance are classified as cyclical-chelators, providing the greatest stability and therefore the safest for use. This is derived from the number of complexing ligands and ionic character of the agent. Magnevist, and MultiHance are ionic linear chelators, while Omniscan and OptiMARK are linear non-ionic chelators. Figure modified from (2).
The agents deemed safe for human use, and thereby approved for use by the FDA, are based on either diethylene triamine pentaacetic acid (DTPA) or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). Both of these materials occupy 8 of the gadolinium(III) binding sites, leaving a single site for water interaction. The limitation of binding sites required to stabilize the ion for in vivo use is to the detriment of the ions use as a paramagnetic $T_1$-w contrast agent. For example the longitudinal relaxivity of the commonly used *Dotarem*, is $R_1 = 3.1 \text{ (mM} \cdot \text{sec)}^{-1}$ at 1.41 T (10).

**Table A.1 - Gd-CA Properties; 37° C**

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Trade name</th>
<th>$\log K_{eq}$ M$^{-1}$</th>
<th>$T_{1/2}$</th>
<th>Osmolality (Osmol/kg)</th>
<th>Viscosity (cP)</th>
<th>$R_1$ (mM·sec)$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gadoteridol</td>
<td>ProHance</td>
<td>23.8</td>
<td>1.3 d</td>
<td>1.91</td>
<td>3.9</td>
<td>3.7</td>
</tr>
<tr>
<td>Gadodiamide</td>
<td>Omniscan</td>
<td>16.9</td>
<td>&lt; 2 s</td>
<td>1.90</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Gadopentetate</td>
<td>Magnevist</td>
<td>25.8</td>
<td>&lt; 2 s</td>
<td>5.85</td>
<td>&gt; 30</td>
<td>3.8</td>
</tr>
<tr>
<td>Gadoterate</td>
<td>Dotarem</td>
<td>25.8</td>
<td>95 d</td>
<td>4.02</td>
<td>11.3</td>
<td>3.5</td>
</tr>
</tbody>
</table>

General features of Gd-chelates are listed above. The $T_{1/2}$ dissociation times are for solutions at acidic pH, where d is days and s is seconds; where macrocycles demonstrate reduced dissociation proclivity. In vivo the situation is more complex, as pH is generally higher and the presence of competing endogenous ions are heterogeneous. Osmolality measurements were conducted at 1.0 M of agent.

Consolidated and adapted from (11).
A.3 - Gd-CA Applications and Cellular Imaging

Gadolinium(III) based contrast agents have found use in a wide variety of clinical applications; particularly for the detection, diagnosis and staging of heart disease, cancers and neurological diseases.

Neurological imaging applications have found great use for gadolinium(III) contrast. Initial diagnosis sensitivity is enhanced when using conventional Gd-CA (12, 13). The agents benefit diagnosis through their accumulation in extracellular spaces allowing for visualization of disruptions of the blood brain barrier (BBB). Further, the CA can be used to monitor the course of β-interferon treatment (14, 15).

A.4 - Gd-CA Limitations

There exist several significant difficulties when using conventional Gd-chelates to enhance tissue contrast. The agents require much higher concentration than radioisotope or optical probes used for (primarily tumor) detection, often in the micromolar range (16). This is a problem resulting from the generally low sensitivity of MR. Attempts to overcome this problem have largely centered on nanoparticle and polymer platforms that seek to deliver higher local concentrationos of Gd.

Conjugation of Gd to larger molecular weight structures, such as proteins (17-19) or dendrimers (20), or as particles (21), is an approach that seeks to effectively increase the rotational correlation time of the Gd-complexes. Another strategy is for the encapsulation of Gd within a nanocarrier to amplify local concentration (and therefore
signal), using liposomes (22), polymersomes (23) or micelles (24). There is often a tradeoff with both approaches that result in either long retention times (and fears of toxicity) or decreased access to tissues or pathological sites (due to pharmacological factors).

A more recent approach, generally seen as suitable only for research applications, is to modify chelates such that they are able to access and accumulate intracellularly. The main focus of this strategy has been to use individual Gd-chelates synthesized with highly cationic polypeptides (such as the HIV-Tat or polyarginine sequences). These chelates are able to gain access to the cellular compartment. The accumulation of chelates was not found to have appreciable toxicity on cultured cells, however application in vivo has not occurred (25, 26).
A.5 - References


