Developing and Characterizing a Multifunctional Iron Oxide Nanoparticle for Cancer Therapy

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Abstract

Nanoparticles (NPs) are ideal drug carriers because of their small, biocompatible size and high surface to volume ratio. Therapeutic, targeting, and diagnostic moieties can be attached to the NP simultaneously to create a novel “multifunctional” therapeutic agent. Herein, efforts toward the synthesis of poly(ethylene glycol) (PEG) and silica-coated iron oxide (Fe\(_x\)O\(_y\)) NPs that can be functionalized with anti-tumor and molecular targeting agents are described. Fe\(_x\)O\(_y\) is a superparamagnetic T2 magnetic resonance imaging (MRI) contrast agent. MRI can be used as a diagnostic tool to track Fe\(_x\)O\(_y\) NPs in vivo. PEG and silica have uniform surface chemistry that is ideal for 1) affording biocompatibility, 2) alleviating aggregation of Fe\(_x\)O\(_y\) NPs, and 3) serving as a scaffold for future functionalization of the nanovehicle. A variety of synthetic methods were investigated to deposit a thin (1-10 nm) layer of silica, PEG, or a mixed silica-PEG interface on commercially-synthesized (Ferrotec EMG 304 ®)10 nm superparamagnetic iron oxide (SPIO) cores. Surface-modified products were characterized with FT-IR spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS), and MRI relaxivity studies. FT-IR and TEM confirmed successful PEGylation of SPIOs with a PEG-silane species. Aggregation and polydispersity were key issues in the silica-modification syntheses. The smallest (15-20 nm overall particle size, 1-3 nm silica layer), most monodisperse silica-coated SPIOs were produced using a microemulsion synthesis with a short (1 hr) reaction time. MRI relaxivity studies of bare and surface-modified SPIOs were able to quantify the T2-MRI contrast effect of NPs. Surface-modified SPIOs were quantified to have less contrast than bare SPIOs, which appeared to result from a combination of the changing surface chemistry and increasing size of the NPs.
Acknowledgments

“And the day came when the risk to remain tight in a bud was more painful than the risk it took to blossom.” – Anais Nin

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1 Introduction

At the turn of the twentieth century, Nobel Prize Laureate Dr. Paul Erlich popularized the idea of a “magic bullet” for treating disease\(^1\). If a compound could be synthesized or found to selectively target diseased cells in the body, Erlich reasoned, a toxin could be delivered with this compound to selectively destroy those - and only those - diseased cells. A century later, we are making effort toward Erlich’s concept of a “magic bullet” therapy. Specifically, the goal of this project is to build a multifunctional, targeted therapy for pancreatic cancer, an aggressive form of cancer with an average survival rate of less than one year and no effective therapies\(^2\).

Nanoparticles (NPs) are ideal platforms for building a multifunctional therapeutic agent because their small size enables cell entry for molecular targeting and there are well-studied synthetic pathways to conjugate anti-tumor moieties onto their surface\(^3\). The aim of this study is to build on previous work\(^4,5\) to optimize the synthesis of a NP with a superparamagnetic iron-oxide (SPIO/Fe\(_x\)O\(_y\)) core, which will be conjugated to a monoclonal antibody (mAb) and boron-loaded polypeptide, poly D-glutamate: D-lysine (poly-GL), to target (via specific antigen-antibody interactions) and kill (via boron neutron capture therapy\(^6\)) pancreatic tumor cells, respectively (Figure 1). The SPIO core is important because the nanoparticle can serve as a magnetic resonance imaging (MRI) contrast agent, making it a valuable diagnostic tool in addition to its role as a therapeutic agent (sections 1.1 and 1.2).

![Figure 1](image_url). Au NP with iron-oxide core functionalized with Poly-GL and tumor-targeting antibodies via an Avidin-Biotin linkage.
To this aim, Zhou and Yayla have already investigated the direct coating of SPIOs with a thin layer of gold (Au), which provides thiol (-SH) linkers for conjugating the mAb and Poly-GL (Figure 1). Both reported limited success, citing presence of uncoated SPIO cores as the main concern. The focus of this study is to pursue alternative synthetic routes to attain a protective coating of the SPIO cores for future functionalization. First, in continuation with investigations by Yayla, a synthesis for silica-coated SPIO cores is being investigated. With versatile silanol (-Si-OH) attachment sites on the surface, silica can act as a precursor for a layer of gold and/or provide a scaffold for direct conjugation with ligands. Second, poly(ethyleneglycol) (PEG) is also being investigated as a scaffold and protective layer. Not only is PEG a well-known agent for improving blood half-life due to its hydrophilicity, but its terminal hydroxyl groups can also be conjugated with therapeutic and targeting groups. Figure 2 is an overview of both synthetic approaches.

Figure 2. 10 nm SPIO cores are (A) coated with a silane-PEG species and (B) coated with silicon dioxide (silica) using tetraethylorthosilicate (TEOS), a silica-precursor.

An additional goal of this study is to quantitatively characterize and monitor if/how MRI contrast of SPIO cores changes upon synthetic modification because size and surface chemistry is likely
to influence efficacy of the contrast agent (section 1.2.2). A theoretical and literature overview of MRI and molecular-targeting SPIO contrast agents is presented in the following sections.

1.1 MRI Theory\textsuperscript{11,12,13}

MRI relies on principles of nuclear magnetic resonance (NMR) to produce high-contrast and high-resolution images of soft tissue inside the body non-invasively, making it a powerful diagnostic technique. In this overview, the principles of NMR relevant to MRI will be presented followed by a discussion about how the NMR signal is collected and spatially encoded to create a MR image.

1.1.1 NMR

Nuclei with an odd number of protons, neutrons, or both are considered “NMR active” because they have a non-zero value of an intrinsic quantum mechanical property called spin, which gives rise to non-degenerate spin energy states in the presence of a magnetic field. Notably, these spin states are quantized and the number of states is given by:

\[ \text{# energy states} = 2S + 1 \quad (1) \]

where \( S \) is the spin angular momentum quantum number. Nearly every element on the periodic table has some NMR-active isotope (i.e. \( S \neq 0 \)), but often the isotopic abundance is not high enough to be detected. \(^1\text{H}\), which has \( S = \frac{1}{2} \), is used in MR imaging not only because of its natural isotopic abundance (99.9%), but also because of its molecular abundance in water (\( \text{H}_2\text{O} \)), which comprises about 60% of the human body\textsuperscript{11}. According to equation (1), a hydrogen proton has two spin states (designated as \( -\frac{1}{2} \) and \( +\frac{1}{2} \)) that lose their degeneracy in a magnetic field. The frequency associated with the quantized energy difference between the two states is called
the Larmor frequency ($\omega$) and it varies with the strength of the external magnetic field ($B_0$) and the gyromagnetic ratio ($\gamma$), which is a fixed proportionality constant specific to each nucleus\footnote{For hydrogen, $\gamma = 42.6$ MHz/Tesla. NMR spectrometers of different magnetic field strengths are often designated as 400MHz, 600 MHz, etc. This refers to the Larmor frequency of the proton calculated using equation (2).}:

$$\omega = \gamma B_0$$  \hspace{1cm} (2)

A NMR signal is produced from the interaction of electromagnetic energy at the appropriate Larmor frequency (fortunately in the non-ionizing radiofrequency (RF) range) with nuclei in each spin state. In order to provide a more tangible explanation of this phenomenon, it is helpful to invoke the classical (Newtonian) treatment of spin for hydrogen nuclei.

In the classical model, hydrogen nuclei may be assumed to be “spinning” about their own axes, each producing a magnetic dipole moment. In the absence of $B_0$, magnetic dipole moments point in random directions and cancel each other out, producing zero bulk magnetization. On the other hand, in the presence of $B_0$, the magnetic dipole moments of hydrogen nuclei align with or against $B_0$ – the two quantized spin states predicted with the quantum mechanical treatment of spin above (Figure 3).

\begin{figure}[ht]
\centering
\includegraphics[width=0.5\textwidth]{diagram.png}
\caption{In the absence of $B_0$, zero net magnetization is produced. In the presence of $B_0$, nuclei align with or against $B_0$.}
\end{figure}

Nuclei that have their magnetic dipole moments aligned with $B_0$ are in the lower energy spin state and those with their magnetic dipole moments against $B_0$ are in the higher energy spin state.
Due to a slight excess of nuclei in the lower spin state at equilibrium\(^2\), there is a *longitudinal* bulk magnetization vector \((M_0)\) in the direction of \(B_0\) that is a vector sum of individual nuclear "spins" precessing *out of phase* around the \(B_0\) axis at the Larmor frequency (Figure 4).

![Figure 4](image1). Precession of nuclear spins at Larmor frequency around \(B_0\) axis produces net longitudinal magnetization \((M_0)\). Because the precession is out of phase, there is no net magnetization in the transverse (x-y) plane.

To produce and detect a NMR signal, however, requires that the longitudinal magnetization be perturbed from its equilibrium state along the \(B_0\) axis into a transverse plane where there is a RF receiver coil that can monitor the precession of bulk magnetization as it returns to equilibrium. This is achieved by applying a RF pulse perpendicular to the longitudinal magnetization in *resonance* at the Larmor frequency (Figure 5).

![Figure 5](image2). Application of a RF pulse perpendicular to \(M_0\) at its Larmor frequency "flips" \(M_0\) into the transverse (x-y) plane.

---

\[^2\] The relative abundance of nuclei in each spin state at equilibrium can be derived using the Boltzmann Distribution:

\[
\frac{N_{\text{upper}}}{N_{\text{lower}}} = e^{-\frac{\Delta E}{kT}}
\]

where \(N_{\text{upper}}\) and \(N_{\text{lower}}\) correspond to the number of nuclei in the higher and lower energy spin states, respectively; \(\Delta E\) is the difference in energy between the spin states; \(k\) is the Boltzmann constant; and \(T\) is the temperature. Because \(\Delta E\) is small, the populations of nuclei in each spin state are nearly identical with a slight excess (~ ten in a million) of nuclei in the lower energy spin state. Because the NMR signal arises from the RF energy absorbed *and* emitted by nuclei that are transitioning between the two spin states, the ten in a million excess of spins is critical to produce a non-zero signal. The ten in a million excess of spins also explains the low sensitivity of NMR/MRI.
After the RF pulse, bulk magnetization precessing *out of phase* around $B_0$ also begins to precess *in phase* around the smaller magnetic field associated with the RF pulse, resulting in a spiral motion of the longitudinal magnetization into the transverse plane. In a rotating frame of reference (Figure 5), the longitudinal magnetization “flips” into the transverse plane. How far it flips away from the $B_0$ axis (i.e. the flip angle $\theta$ in Figure 5) is a function of the strength and the duration of the RF pulse; a 90° RF pulse flips *all* longitudinal magnetization into the transverse plane.

After completion of the RF pulse, transverse magnetization decays through T1 and T2 relaxation processes (discussed below) to re-establish equilibrium along the $B_0$ axis. As relaxation takes place, the magnetic field oscillating at the Larmor frequency in the transverse plane induces current in a RF receiver coil, which produces a decaying sinusoidal signal called a free induction decay (FID). FIDs, as discussed in 1.1.3, are spatially encoded and Fourier transformed to produce an MR image.

### 1.1.2 T1 and T2 Relaxation

A 90° RF pulse tips bulk magnetization into the transverse plane where it precesses *in phase*. What happens after the RF pulse is turned off? Thermodynamically, a system wants to re-establish equilibrium and return to its lowest energy state. Two separate, simultaneous processes occur after application of the RF pulse to return the spin state system to equilibrium:

1. Nuclei return to their lowest spin energy state
2. Nuclear spins precessing in phase get out of phase with each other

In the classical vector model this corresponds, respectively, to:

1. Recovery of the longitudinal component of bulk magnetization along the $B_0$ axis
2. Loss, or dephasing, of the transverse component of bulk magnetization
T1 and T2 relaxation are the terms used to describe the recovery of longitudinal magnetization (Mz) and decay of transverse magnetization (Mxy), respectively, in the classical model (Figure 6).

Mathematically, T1 and T2 are time constants in exponential functions (the Bloch equations) (3, 4) that characterize the rate of the two independent processes:

\[ M_z(t) = M_{z(0)} \left( 1 - e^{-\frac{t}{T_1}} \right) \quad (3) \]
\[ M_{xy}(t) = M_{xy(0)} \left( e^{-\frac{t}{T_2}} \right) \quad (4) \]

By the definition of an exponential time constant, T1 is the time it takes for 63% of Mz to return to its equilibrium magnetization Mz(0) along the B0 axis (1.3). On the other hand, T2 is the time constant of a decaying exponential so it is the time it takes to reach 37% (or lose 63%) of the initial transverse magnetization, Mxy(0). Both processes, occurring simultaneously, are graphed together in Figure 7. Note that in Figure 7, the growth and decay of each exponential process is not the same. T2 decay occurs 5 to 10 times faster than T1 recovery.

Figure 6**. (Rotating Frame) After the RF pulse is turned off, the transverse vector Mxy begins to decay (T2 relaxation) and the longitudinal component Mz recovers (T1 relaxation). The two processes occur simultaneously, but are independent of each other.

Figure 7**. The rate of decay of transverse magnetization (T2) is faster than recovery of longitudinal magnetization (T1). Relative T1 (red) and T2 (blue) relaxation times are indicated by vertical lines.
Because NMR signal intensity is a function of relaxation time, natural contrast in MR images arises from the unique T1 and T2 times of different tissues in the body. Moreover, the contrast among tissues can be optimized by adjusting parameters in MR pulse sequences (section 1.1.3) and/or by using contrast agents (section 1.2). Table 1 lists typical T1 and T2 times of several tissues in the body. In order to understand why particular tissues have smaller or bigger T1 and T2 values, it is important to briefly discuss the physical mechanisms responsible for T1 and T2 relaxation.

Table 1. T1 and T2 relaxation times at 1.5 T

<table>
<thead>
<tr>
<th>Tissue</th>
<th>T1 (ms)</th>
<th>T2 (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrospinal Fluid (CSF)</td>
<td>2400</td>
<td>600</td>
</tr>
<tr>
<td>Fat</td>
<td>270</td>
<td>80</td>
</tr>
<tr>
<td>Cartilage</td>
<td>1030</td>
<td>40</td>
</tr>
</tbody>
</table>

Just as its name implies, spin-lattice (T1) relaxation involves transfer of energy between excited nuclei spin states and the surrounding “lattice” of other nuclei in the local environment to restore the distribution of spin states predicted by the Boltzmann Distribution. Energy transfer is most efficient, and thus most significant, when the natural motional frequencies[^3] of nuclei are at the Larmor frequency. Natural motional frequencies arise from random translational, vibrational, and rotational movement of molecules in solution and can generate an oscillating magnetic field due to the movement of charged particles. When the frequency of the oscillating field is equal to the energy difference between states (i.e. Larmor frequency), a non-radiative form of stimulated emission allows excited spin states to relax down to their ground state. Spontaneous emission of

[^3]: Natural motional frequency, which is influenced by the chemical environment of the hydrogen nuclei, should not be confused with precessional frequency, which is a function of the Larmor frequency and magnetic field strength (see equation 2).
excited spin states is not significant in NMR because it is proportional to the third power of the emission frequency, which is in the low radiofrequency range.

Because T1 depends on molecular movement, it is influenced by the mobility of the lattice. Water protons in Cerebrospinal Fluid (CSF), a colorless fluid around the brain and spinal cord, for example, have very high natural motional frequencies and water protons in cartilage, a compact tissue, have very slow motional frequencies. In both cases, the motional frequencies do not match the Larmor frequency, resulting in inefficient (slow) T1 relaxation. Fat, on the other hand, has protons with natural motional frequencies very close the Larmor frequency, causing efficient (fast) T1 relaxation (Table 1).

Unlike spin-lattice relaxation which involves a change in energy of spin states, spin-spin (T2) relaxation describes the change in phase of magnetic moments after the application of the RF pulse. In spin-spin relaxation, which is often referred to as an entropic process, nuclei with magnetic moments that are lined up in the transverse plane after the RF pulse get out of phase due to a) local spin-spin interactions and b) inherent inhomogeneity in the external magnetic field. In the case of spin-spin interactions, small magnetic fields created by proximal spins aligned with or against $B_0$ increase or decrease the overall magnetic field “felt” by individual spins, respectively, causing spins to get out of phase over time as they take on a range of precessional frequencies according to equation (1). In addition, there will always be some variation in the external magnetic field that causes protons in one region to precess at a different Larmor frequency than in another region, contributing to spin dephasing. It is possible to measure the relaxation time due solely to spin-spin interaction (T2) or both spin-spin and external magnetic field inhomogeneity ($T2^*$). T2, a fixed value for a specific tissue, and $T2^*$ are related through the deviation present in the external magnetic field ($\Delta B$) and the proton gyromagnetic ratio ($\gamma$):
\[
\frac{1}{T_{2*}} = \frac{1}{T_2} + \gamma \Delta B \tag{5}
\]

Because T2* takes into account \(\Delta B\) and spin-spin interactions, \(T_2 > T_{2*}\).

Since T2 time is a function of how fast proton spins dephase due to interactions between spins, it depends on the molecular environment of protons. In pure water, for example, the interatomic distance between protons is relatively large due to the geometry of the water molecule and spin-spin interaction is therefore minimal. Generally, the more compact the tissue, the more spin-spin interactions take place, leading to shorter T2 times. This explains the T2 times for CSF > Fat > Cartilage in Table 1.

1.1.3 Pulse Sequences & Image Construction

In order to improve contrast and obtain spatial information from multiple FIDs, specific MRI pulse sequences – protocols that specify the timing and strength of RF pulses (and often magnetic field gradients) – are used to collect signal during an MR imaging session. The two main parameters of a pulse sequence are Time to Repetition (TR) and Time to Echo (TE). TR is the time between consecutive excitation RF pulses (usually 90°) and TE is the time between the excitation pulse and collection of signal from a FID called an echo\(^4\). The TR and TE intervals for a common MR pulse sequence called a spin-echo are shown in Figure 17 in section 1.2.3.

It is important to remember that unlike the fixed values\(^5\) T1 and T2, TR and TE can be adjusted by the operator. Thus, both sets of variables along with the number of mobile protons, \(N(H)\), factor into overall signal intensity:

\[
\text{Signal Intensity} \propto N(H) (e^{-\frac{TE}{T_2}}) (1 - e^{-\frac{TR}{T_1}}) \tag{6}
\]

\(^{[4]}\) Spatial-encoding steps (Figure 8) do not allow measurement of FID immediately after the RF pulse so signal is usually collected at some time (TE) after the pulse. In order to do this, often refocusing 180° RF pulses are applied to generate an “echo” of the original FID. See discussion of the spin-echo pulse sequence in section 1.2.3.

\(^{[5]}\) Except when T1 and T2 times are shortened with contrast agents (see section 1.2)
By appropriately adjusting TR and TE, it is possible to see from equation (6) that more or less weight can be given to signal from T1 or T2 relaxation processes. So-called T1-weighted images generally have a short TE and short TR (TE < 30 ms, TR < 1000 ms) to maximize signal from longitudinal relaxation while T2-weighted images have a long TE and long TR (TE > 80, TR > 2000 ms) to capture signal largely from transverse relaxation\(^6\).

Before FID signals obtained using a specific MR pulse sequence can be processed into an image, they need to be spatially encoded. That is, there needs to be a way to specify the origin of the received signal in three-dimensional space. This is achieved by applying gradients – linearly varying magnetic fields – in three dimensions (Figure 8).

![Figure 8](image)

**Figure 8**. Spin-echo pulse sequence diagram and associated gradients denoted as \(G_z\) (Slice-Select), \(G_y\) (Phase-Encoding), and \(G_x\) (Frequency-Encoding). The rectangle is the symbol for a linearly varying gradient. The unique symbol for the phase-encoding gradient \((G_y)\) indicates the multiple phase-encoding steps that must be made in a single acquisition (a separate TR is needed for each phase-encoding step).

Initially, a gradient coil varies the magnetic field along one of the three dimensional axes (x, y, or z) during the RF excitation pulse. Because the Larmor frequency varies with magnetic field strength \((\omega = \gamma B_0)\), the gradient \((G)\) creates a position-dependent spin \((\omega^*)\) along the axis \((z)\):

\[
\omega^* = \gamma(B_0 + zG) = \omega_0 + \gamma zG \quad (7)^{12}
\]

\[
\therefore z = (\omega^* - \omega_0)/(\gamma G)
\]
This gradient, applied along the z axis in Figure 8, is called a *slice-select* gradient because if a RF pulse with a narrow bandwidth is applied to a sample, only nuclei in a particular “slice” of the linearly varying $B_0$ will have the appropriate Larmor frequency to be excited. The *phase-encoding* gradient (applied after the excitation RF pulse) and the *frequency-encoding* gradient (applied during the readout, or, time the echo is formed) are the other two gradients applied along the remaining axes (y and x in Figure 8), respectively, allowing the spatial information to be collected along these axes in the same manner as outlined in equation (7). The final step is to Fourier transform the spatially-encoded two-dimensional array of FID signals from the time to frequency domain (note that in equation (7) position corresponds to frequency) to create a 2D MR image.

### 1.2 Contrast Agents & Molecular MRI

Since the development of MRI in the 1970s, chemical compounds clinically referred to as “contrast agents” have been used to selectively improve contrast of specific tissues in anatomical MR scans. In general, clinical contrast agents work by generating fluctuating magnetic fields to shorten relaxation times of water in certain tissues, creating brighter signal in T1-weighted scans and darker signal in T2-weighted scans according to equation (6). Although a contrast agent can usually shorten both T1 and T2 times, it is classified as either a T1 or T2 contrast agent depending on which type of contrast it produces more effectively. Chelates of paramagnetic Gd(III) and polymer-coated SPIO particles are common T1 and T2 clinical contrast agents, respectively. SPIOs in particular, due to their nanomolar sensitivity\(^6\) and high-contrast capabilities, have emerged as attractive platforms to build nano-scale probes that have the potential to make MRI a real-time diagnostic tool at the cellular and molecular level\(^17,18\).

\(^6\) Gadolinium detection is in in micromolar range\(^17\)
The emerging field of “molecular MRI” has attracted nanotechnology and biotechnology researchers to experiment with SPIO NP-based molecular probes. In one successful example of imaging breast cancer cells, SPIO NPs were conjugated with the HER2/neu antibody specific for antigens expressed by breast cancer cell lines. A T2-weighted ex vivo “phantom” MRI study showed noticeable negative contrast (i.e., signal darkening) in the cancer cell lines treated with the SPIO-conjugated-HER2/neu antibody, highlighting the ultra-sensitive detection capabilities of SPIOs in MRI (Figure 9).

In addition, conjugated SPIOs were found to be biologically nontoxic at the test concentrations, making them an even more attractive probe for biomedical applications. The following sections provide a chemical-physical background of SPIOs and their T2 relaxation mechanism.

1.2.1 Superparamagnetic Behavior

SPIO contrast agents are colloidal suspensions of nano-size SPIO crystals. The most common SPIOs are magnetite (Fe$_3$O$_4$) and maghemite ($\gamma$-Fe$_2$O$_3$). Ferromagnetic materials, bulk magnetite and maghemite are composed of magnetic domains (Weiss domains) separated by Bloch wall transition regions. Each domain is uniformly magnetized due to the alignment of
magnetic moments arising from unpaired electron spins in the SPIO crystal lattice\footnote{This is a consequence of long-range ordering phenomena governed by quantum mechanics.}. When an external magnetic field is applied, the domains orient parallel to the field, producing a high degree of magnetization due to the alignment of already magnetized domains (Figure 10).

An interesting phenomenon occurs for nano-size magnetite and maghemite crystals. Formation of Bloch walls becomes thermodynamically unfavorable for particles ~ 14 nm and below and each crystal acts as a single domain with superparamagnetic behavior\footnote{This sets a functional limit on the size of iron oxide cores in order for them to be superparamagnetic (<14 nm). Because iron oxide NPs are usually synthesized with a polymer coating, however, SPIOs are typically characterized in terms of their overall particle diameter, which can range from tens of nanometers to micrometers.}. As the name implies, superparamagnetic crystals have enhanced paramagnetic behavior. That is, they have a greater magnetic susceptibility (the degree of magnetization induced by external magnetic field) than

---

\textbf{Figure 10}\textsuperscript{21}. Bulk ferromagnetic material consists of many magnetic domains. When \( B_0 = 0 \), the domains are oriented randomly, producing zero net magnetization (left). When \( B_0 \) is turned on, domains orient parallel to the field and net magnetization is produced (right).

\textbf{Figure 11}\textsuperscript{21}. Magnetic behavior of (A) paramagnetic and (B) ferromagnetic material in an external magnetic field (the \( B \) vs. \( M \) curve for a superparamagnetic material is drawn in red). The curves represent induced magnetization \( M \) as a function of the applied field \( B \). Paramagnetic material has non-interacting spins (lower left) while ferromagnetic/superparamagnetic material possesses strongly interacting spins (lower right).
paramagnetic materials. This is due to the fact that magnetic moments arising from unpaired electron spins in paramagnetic materials align with the external magnetic field independently (as opposed to positive interaction among domains in ferromagnetic/superparamagnetic materials), resulting in lower susceptibilities than superparamagnetic materials (Figure 11). It is notable in Figure 11 that ferromagnetic materials can possess a large magnetization even in the absence of an external field (i.e. when $B = 0$ there is a non-zero value of $M$). Both superparamagnetic and paramagnetic materials differ in this regard and do not have any magnetization remaining once removed from the external field. Instead, thermal energy maintains random orientation of the domains in SPIOs, preventing their aggregation when the field is turned off.

### 1.2.2 SPIO-Induced T2 Relaxivity

SPIO magnetic moments induced by the external magnetic field perturb the T2 relaxation of protons in their vicinity. Because magnetic fields are distance-dependent, protons diffusing near SPIOs experience more rapid dephasing of their spins than protons further away, resulting in a shorter T2 time for the nearby protons that corresponds to a darker region in a T2-weighted MR scan (Figure 12).

![Figure 12](image-url)  
*Figure 12*: (Left, lower panel) MR image of tissue without SPIO contrast agent; (Right, lower panel) MR image of tissue in the presence of SPIO particles.
A parameter called the molar relaxivity is generally measured (section 1.2.3) to evaluate the efficiency and effectiveness of contrast agents like SPIOs. The molar relaxivity \( r \), expressed in units of mM\(^{-1}\)s\(^{-1}\), is a contrast agent-specific parameter that takes into account the contrast agent concentration \([CA]\) and relaxation rate \( R_2 \) or \( R_1 \)\(^{21}\). T2 molar relaxivity \( r_2 \) is defined as:

\[
r_2 = \frac{R_2}{[CA]} \quad \text{where} \quad R_2 = \frac{1}{T2}
\]  

(8)

Table 2 lists the \( r_2 \) values for several SPIO-based clinical contrast agents.

**Table 2**. Size, composition, and molar relaxivity value for conventional SPIO contrast agents.

<table>
<thead>
<tr>
<th>Name</th>
<th>SPIO Core Size (Total Size)</th>
<th>Polymer Coating</th>
<th>( r_2 ) (mM(^{-1})s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feridex ®</td>
<td>5-6 (80-150) nm</td>
<td>Dextran</td>
<td>100</td>
</tr>
<tr>
<td>Resovist ®</td>
<td>4.2 (62) nm</td>
<td>Carbodextran</td>
<td>151</td>
</tr>
<tr>
<td>Sinerem ®</td>
<td>4-6 (20-40) nm</td>
<td>Dextran</td>
<td>53</td>
</tr>
</tbody>
</table>

The range of values in Table 2 reveals relaxivity is a function of many variables, including size, composition, and surface chemistry of the contrast agent. Similar relaxivity values, for example, in the range of 70-75 mM\(^{-1}\)s\(^{-1}\) were reported for both 10 nm SPIO NPs coated with a hydrophilic polymer and 30 nm SPIO NPs coated with a hydrophobic polymer, which highlights the complex interplay of size and coating composition on overall relaxivity\(^{22}\). It should also be noted that the expected linearity of \( R_2 \) with \([CA]\) according to equation (8) depends on the spatial distribution and the aggregation state of particles and may not always be observed\(^{7,21,23}\).

There are two major physical mechanisms that govern relaxivity: inner-sphere and outer sphere relaxation\(^{24}\). Inner sphere interactions take into account direct bonding and coordination between the solvent and contrast agent. Outer sphere interactions, on the other hand, characterize long-range interactions due to diffusion of solvent in the magnetic field generated by the contrast agent. Contrast agent-induced relaxation rates for T1 and T2 processes can be written in terms of
outer and inner sphere interactions. Unlike T1-based Gd(III) contrast agents, SPIOs do not have coordination sites for water, making inner sphere relaxation negligible. Thus, the relaxation rate for protons diffusing in the proximity of SPIOs is largely due to outer sphere relaxation\(^{25}\).

To gain insight into outer sphere relaxation, LaConte et al. developed a Monte Carlo model to simulate the independent effects of the distance of water from the SPIO center (water exclusion radius) and retention of water in a hydrophilic coating around the SPIO core (slow compartment radius) on the relaxivity of SPIOs. Results from the simulation show that as the water exclusion radius increases there is a sharp decrease in \(R_2\), but as the slow compartment radius increases there is near-linear increase in \(R_2\) (Figure 13)\(^{26}\).

These opposing trends make chemical sense: the farther away water molecules are (exclusion radius), the more excluded they are from the distance-dependent dephasing induced by the magnetic moment of the SPIOs. On the other hand, the thicker the hydrophilic coating layer, the more intermolecular interactions there are to retain water for a longer time in the proximity of the SPIO core (i.e., diffusion coefficient of water becomes slower). Notably, when \(R_2\) was plotted as a function of both slow compartment and exclusion radii, it better approximated empirical data (vs. individual models), suggesting that competing factors determine the overall \(R_2\) value\(^{26}\).
In addition to empirical-modeling approaches, outer-sphere theoretical calculations have been performed. A derivation of outer sphere theory\textsuperscript{27} yielded an expression for the T2 relaxation rate ($1/T2$), which is simplified below,

\[
\frac{1}{T2} \propto \mu^2 \gamma_1^2 N_A \left( \frac{M}{rD} \right) j_n(\omega, \tau)
\]  

(9)

where $\mu$ is the magnetic moment of the NP, $\gamma_1$ is the gyromagnetic ratio of protons in water, $N_A$ is Avogadro’s number, $M$ is the molarity of the NP, $r$ is its radius, $D$ is the relative diffusion of the particle and the water, and $j_n(\omega, \tau)$ are Fourier transformed correlation functions (spectral density functions) of particle-water magnetic dipole interactions due to diffusion\textsuperscript{18}.

Previous studies have taken advantage of the fact that $R_2 \propto \mu^2$ to synthesize SPIOs with bigger magnetic moments in order to increase $R_2$. In particular, there are two common ways to maximize $\mu$: 1) increase the size of the SPIO core and 2) dope the SPIO crystal lattice with paramagnetic elements\textsuperscript{18}. The $\mu$ size-dependence arises from misaligned spins that form around the surface of smaller NPs and decrease the overall magnetic moment of the crystal. As the diameter of the particle increases, this “spin-canting effect” is less apparent, resulting in an increased $\mu$ (Figure 14)\textsuperscript{18,19}. Doping the SPIO lattice with magnetically susceptible elements such as Mn, Co, and Ni has also been shown to increase $\mu$ by altering the electron spin configuration in the lattice\textsuperscript{19}.

\textbf{Figure 14.} (A)\textsuperscript{16} Spin-canting decreases overall $\mu$ for smaller SPIO particles (B)\textsuperscript{19} Relaxivity increases with size of Fe$_3$O$_4$ (MEIO) NPs
In our study, commercially-synthesized 10 nm Fe$_x$O$_y$ NPs are used for the SPIO cores. Although a co-precipitation synthesis$^{28}$ for Fe$_x$O$_y$ SPIOs has been previously explored$^{4,5}$, the commercial product offers a more convenient source of SPIO cores to optimize surface coating procedures. These 10 nm cores are also ideal for biocompatibility reasons. Because 50-100 nm NPs are ideal for cellular uptake and selective biodistribution in the body$^{29}$, a 10 nm core offers ample “building room” for coating and future functionalization. With this in mind, increasing the size of the SPIO core to improve relaxivity is undesirable.

Returning to equation (9), it is also apparent that $R_2$ is a function of Fourier transformed correlation functions, $j_n(\omega, \tau)$. These correlation functions parameterize the random molecular motions that lead to fluctuating magnetic fields and ultimately cause spin dephasing. From the correlation function, a correlation time constant ($\tau$) can be calculated, which is a measure of how fast (small $\tau$) or how slow (large $\tau$) the fluctuations are taking place (Figure 15).

![Figure 15](image)

**Figure 15**$^{21}$. (A) The effective magnetic field $B_e(t)$ experienced by a water molecule due to molecular motions varies over time; (B) A time correlation function $G(\tau)$ of fluctuations in (A) produces an exponential decay function from which the correlation time, $\tau_c$, can be calculated; (C) The spectral density function $j(\omega)$ is twice the Fourier transformed correlation function.

For SPIO-induced T2-relaxation, there are three relevant correlation times:$^{21}$ the 1) rotation of magnetization within the SPIO particle ($\tau_N$), 2) rotation of the SPIO particle and magnetization as a whole ($\tau_B$), and 3) diffusional motion of water in the proximity of the contrast agent ($\tau_D = R^2/D$ where $R$ is the radius of the particle and $D$ is the diffusion coefficient of water) as shown in Figure 16.
Because $\tau_D \propto R^2$, larger particles (μm) generally have a long correlation time (i.e., NPs assumed to be static relative to water molecules) and smaller particles (nm) have a short correlation time (i.e. diffusion of NPs is assumed to be very fast). On the basis of size alone, then, it is plausible that coating the iron-oxide cores (i.e. increasing the overall size of the particle) will increase the correlation time and, thus, decrease the diffusion contribution to T2 relaxation.

### 1.2.3 Measuring T2 Relaxivity

According to equation (8), there are two quantities that must be measured to obtain $r_2$: [SPIO] and the T2 relaxation time. The [Fe] in SPIOs is generally used as the molarity concentration value for SPIO-based contrast agents and can be determined spectroscopically. The T2 relaxation time can be obtained from signal intensity measurements of MR images acquired using a spin-echo MRI pulse sequence (Figure 17).
First, a 90° excitation RF pulse flips the magnetization vector into the transverse plane. Recall, immediately after the RF pulse is turned off, the spin vectors are precessing in phase around the B₀ axis. At a certain time τ after the spins get out of phase, a 180° RF pulse is applied. As its name implies, the 180° pulse reverses the spins in the transverse plane by 180°. The 180° pulse refocuses the spins, causing them to rephase as they precess in reverse direction. They reach maximum rephasing at time 2τ, producing a signal called an echo. Multiple 180° pulses can be applied at 2τ intervals, producing an exponentially decaying echo train (Figure 18).

![Figure 18](image)

**Figure 18**. Echo train produced by a dual-echo, spin-echo pulse sequence. T2 is the time constant for the echo train decay; T2* is the time constant for individual echoes.

The decay function can be fit to T2 portion of equation (6) to obtain the time constant, T2, from a linear plot of 1/T2 and ln(SI):

\[
T2 \text{ Signal Intensity} = SI = S_0(e^{-\frac{TE}{T2}}) \tag{10}^{31}
\]

\[
\ln(SI) = -\frac{TE}{T2} + \ln(S_0)
\]

\[
\ln(SI) = \left(-\frac{1}{T2}\right)TE + \ln(S_0)
\]

Ultimately, the SE pulse sequence makes it possible to regain signal lost due to the external magnetic field inhomogeneity. Because T2 relaxation is an intrinsic property, only decay of the signal due to spin-spin interactions is present in the echo signal produced by the 180° refocusing pulse.
2 Materials and Methods

2.1 Functionalization of Fe$_x$O$_y$ NPs

A commercial water-based colloidal Fe$_x$O$_y$ NP solution (Ferrotec EMG 304®, 4.5% v/v, 10 nm average size) was used as the SPIO NP core precursor for Fe$_x$O$_y$ NP functionalization (Fe$_x$O$_y$®) with silica (SiO$_2$) and/or poly(ethylene glycol) (PEG). Figure 19 is a flowchart of the syntheses explored in this study. The viscous EMG 304® colloidal suspension of Fe$_x$O$_y$ NPs was typically diluted to create a 1.3% w/v stock solution (Ferrotec stock) with 18.3 MΩ·cm H$_2$O (nH$_2$O) or absolute ethanol (abs. EtOH).

![Flowchart of surface modifications attempted in this study on commercially-synthesized 10 nm Fe$_x$O$_y$ NPs (EMG 304®). The section within Materials & Methods corresponding to each route is indicated in parentheses.](image)

2.1.1 Fe$_x$O$_y$@SiO$_2$ NPs

Silica-coated Fe$_x$O$_y$ NPs (Fe$_x$O$_y$@SiO$_2$) were prepared using:
1. The Stöber synthesis originally described by Ji et al.\textsuperscript{7} and adapted from Yayla\textsuperscript{5} (Stöber Method, section 2.1.1.1) or

2. A reverse microemulsion modified-Stöber functionalization procedure adapted from Yi et al.\textsuperscript{32}, Deng et al.\textsuperscript{33}, and Narita et al.\textsuperscript{34} (Microemulsion Method, section 2.1.1.2).

### 2.1.1.1 Stöber Method

An aliquot of aqueous Ferrotec stock solution was dissolved in nH\textsubscript{2}O + abs. EtOH + NH\textsubscript{4}OH (Sigma Aldrich 28\% w/v) and sonicated for 2-4 minutes. Tetraethyl orthosilicate (TEOS, Sigma Aldrich, 99.9\%) was then added under continuous stirring over a magnetic stir plate. Amounts of reagents typically used are given in Table 3. Contents were left to stir overnight (~12 hours) and SPIO products were collected over a magnet (Qiagen Cat # 36910). After the Fe\textsubscript{x}O\textsubscript{y} NP product visibly collected (dark brown pellet) next to the magnet, the supernatant was decanted and 2-3 washing steps (re-suspend pellet in abs. EtOH and/or nH\textsubscript{2}O, sonicate, re-collect over magnet) were performed; the washed product was suspended in abs. EtOH.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrotec Stock in nH\textsubscript{2}O</td>
<td>1.00 mL</td>
</tr>
<tr>
<td>nH\textsubscript{2}O</td>
<td>500 μL – 2.60 mL</td>
</tr>
<tr>
<td>Abs. EtOH</td>
<td>7.00 – 9.00 mL</td>
</tr>
<tr>
<td>NH\textsubscript{4}OH (28% w/v)</td>
<td>875 μL</td>
</tr>
<tr>
<td>TEOS (99.9%)</td>
<td>5.00 μL</td>
</tr>
</tbody>
</table>

### 2.1.1.2 Microemulsion Method

Cyclohexane (Mallinckrodt 99.0\%) and Igepal© CO-520 (Sigma Aldrich) were stirred on a magnetic stir plate for 5-10 minutes. Next, an aliquot of the aqueous EMG 304© suspension was weighed and added to the cyclohexane-Igepal mixture, which was immediately sonicated for 3-5
minutes to establish a reverse emulsion phase. NH$_4$OH (Sigma Aldrich 28% w/v) was added to the microemulsion and then left on an overhead shaker for one hour. Over continuous stirring, tetraethyl orthosilicate (TEOS, Sigma Aldrich, 99.9%) was added to the sonicated (3-5 min) NH$_4$OH-microemulsion mixture. Contents were stirred on a magnetic stir plate for 1-24 hours\textsuperscript{[9]} and the reaction was terminated by the addition of an equal volume of 1) abs. EtOH or 2) a solution of 10 μL PEG-silane (Polymer Source, Trimethoxy propyl-terminated PEG methyl ether 420 MW) dissolved in 2.00 mL nH$_2$O + 20 mL abs. EtOH or 3) a solution of 10 μL PEG-silane (420 MW) dissolved in 11 mL abs. EtOH. Products were collected and washed in same manner described in section 2.1.1.1 for the Stöber method. The quantities of reagents typically used are given in Table 4.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexane</td>
<td>10 mL</td>
</tr>
<tr>
<td>Igepal CO-520</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Ferrotec EMG 304®</td>
<td>0.001 – 0.006 g</td>
</tr>
<tr>
<td>NH$_4$OH (28% w/v)</td>
<td>875 μL</td>
</tr>
<tr>
<td>TEOS (99.9%)</td>
<td>5.00 μL</td>
</tr>
</tbody>
</table>

### 2.1.2 Fe$_x$O$_y$@PEG NPs

An aliquot of the aqueous EMG 304® suspension was sonicated (2-5 min) and then stirred with a PEG-silane species (Polymer Source, Trimethoxysilane PEG methyl ether 1050 MW or 420 MW) in abs. EtOH overnight. Alternatively, the PEG-silane species was added in place of TEOS in the reverse microemulsion synthetic scheme outline in section 2.1.1.2. Collection and washing steps for Fe$_x$O$_y$ NPs were performed as described in section 2.1.1.1. A negative control functionalization was also done with a PEG-thiol species (Polymer Source, α-hydroxyl-ω-thiol

\textsuperscript{[9]} Reaction times were varied for different trials
terminated PEG 2300 MW) to investigate efficacy of washing steps. Reagent amounts typically used are given in Table 5.

Table 5. Typical reagent amounts used in the PEGylation of Fe$_x$O$_y$ NPs.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrotec EMG 304®</td>
<td>0.01 – 0.05 g</td>
</tr>
<tr>
<td>PEG-silane 1050 MW</td>
<td>0.02-0.05 g</td>
</tr>
<tr>
<td>PEG-thiol 2300 MW</td>
<td>0.02-0.05 g</td>
</tr>
<tr>
<td>PEG-silane 420 MW**</td>
<td>10 μL, 400 μL</td>
</tr>
<tr>
<td>Abs. EtOH</td>
<td>5 mL</td>
</tr>
</tbody>
</table>

*Approximate range inferred based on “pinch” of viscous EMG 304® added
**10 μL used in microemulsion method, 400 μL used in abs. EtOH functionalization

2.1.3 PEGylation of Fe$_x$O$_y$@SiO$_2$ NPs

Silica and PEG Fe$_x$O$_y$ NP functionalization was performed using the microemulsion reaction scheme described in section 2.1.1.2 with the following alteration: PEG-Silane (Polymer Source, Trimethoxysilane PEG methyl ether 420 MW) was added to the cyclohexane + Igepal + NH$_4$OH reaction mixture (3.5 mL) simultaneously with TEOS (1.6 μL) in 2:1, 4:1, and 8:1 PEG to TEOS volume ratios.

2.2 Physiochemical Characterization of NPs

2.2.1 Transmission Electron Microscopy (TEM)

Morphology and size of distribution of synthesized NPs were examined by electron microscopy (Phillips CM 10, 80 KV accelerating voltage) images. TEM samples were prepared by mounting 1-3 drops of the sonicated nanoparticle solution (dissolved in abs. EtOH or nH$_2$O) on a formvar 400 mesh CU grid (Ladd Research) and allowing it to dry in air. TEM images were generally collected at 34,000× – 64,000× magnifications. A manual procedure was used to measure
particle size: a particle on the imaging screen was translated across the diameter of the nanoparticle and the translation distance was recorded by the instrument.

2.2.2 FT-Infrared (IR) Spectroscopy

IR spectra of Fe\textsubscript{x}O\textsubscript{y}@PEG and Fe\textsubscript{x}O\textsubscript{y}@SiO\textsubscript{2} products and reactants were obtained to indirectly verify silica and/or PEGylation functionalization procedures. FT-IR measurements were carried out using a Perkin-Elmer Spectrum One FT-IR spectrometer with a universal ATR sampling accessory (scanning range: 650-4000 cm\textsuperscript{-1}; number of scans: 64-256; resolution: 2.00 cm\textsuperscript{-1}). To improve S/N\textsuperscript{[10]}, a concentrated slurry of the NP solution dissolved in abs. EtOH was pipetted over the ATR crystal. IR spectra were collected on the solid sample after the EtOH evaporated (2-4 minutes) using air as the background scan (adapted from Yayla\textsuperscript{5}).

2.2.3 Dynamic Light Scattering (DLS)

DLS (Malvern Nano-2S) data were used to obtain, when possible, a size distribution of the bare and functionalized Fe\textsubscript{x}O\textsubscript{y} NPs. Sonicated samples were filtered (2 μm Tuffryn membrane filter) and DLS correlation and size data were obtained using the standard operating procedure (SOP) file FeOx.sop; collection parameters: 173° backscatter measurement, 120s equilibrium time, RI = 2.940, Abs = 0.05 in ethanol at 25° C. Efforts were made to sonicate (2-5 minutes) NP sample solution (dissolved in abs. EtOH or nH\textsubscript{2}O) immediately prior to analysis to limit Fe\textsubscript{x}O\textsubscript{y} NP aggregation.

2.3 Characterization of SPIO MR Contrast Effect (T2 Molar Relaxivity)

\textsuperscript{[10]} S/N ratio can be improved by increasing signal or getting rid of noise. In this case, a concentrated slurry will allow a greater intensity signal, creating a greater difference between the signal and random, unwanted noise, resulting in an increased S/N. Other methods for improving S/N include: ensemble averaging, post-processing modifications (e.g. FT filter, boxcar averaging).
2.3.1 [Fe\(^{2+}\)] Determination

The [Fe] in Fe\(_x\)O\(_y\) NP samples for molar relaxivity calculations were obtained using either UV-Visible Spectroscopy (UV-Vis) or Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES).

2.3.1.1 UV-Vis Colorimetric [Fe\(^{2+}\)] Determination

A spectrophotometric protocol developed by Oca-Cassio et al.\(^{30}\) was adapted to determine [Fe] in Fe\(_x\)O\(_y\) NP samples. In a borosilicate disposable test tube, a dilute aliquot (0.5 – 2 mL) of bare or functionalized Fe\(_x\)O\(_y\) NPs was acid digested with an equal volume of 70% w/w (15.7 M) HNO\(_3\) (EMD Chemicals, ACS grade) overnight to displace oxygen from the iron ions in the crystal lattice. The solution turns from rust-brown to clear colorless or light yellow solution depending on [Fe]. Next, the acid-digested solution was buffered by adding 10% w/v sodium acetate (pKa = 4.76) drop-wise until pH ~ 4-5. An excess of 2% w/v hydroxylamine was then added to reduce all Fe\(^{3+}\) to Fe\(^{2+}\) ions. Finally, an excess of 0.002 M o-phenanthroline (Fisher Scientific, ACS grade), a bidentate ligand that complexes with Fe\(^{2+}\) to form a bright red-orange compound, was added to the solution (Figure 20A & B). UV-Vis spectra (Varian Cary 500 Scan UV-Visible NIR) were collected in the 200-800 nm range using a quartz cuvette (\(l = 1\) cm) to measure the peak absorbance of the complex (\(\lambda \sim 510\) nm).

![Figure 20](image-url) A) Ferrotec dissolved in nH\(_2\)O; B) Ferrotec digested with concentrated HNO\(_3\) and added to an excess of o-phenanthroline; C) The orange-red Fe(II)-Phenanthroline has a visible absorption \(\lambda_{\text{max}}\sim 510\) nm. Using Beer-Lambert’s law, the [Fe] can be determined.
Beer’s Law \( (A = \varepsilon cl) \) and a standard curve\(^{35} \) obtained with known [Fe] (Figure 21) were used to obtain [Fe\(^{2+}\)] in NP samples from UV-Vis peak absorbance measurements. An example tris(1,10-phenanthroline)iron(II) absorbance plot is shown in Figure 20C.

![Absorbance vs. Concentration](image)

**Figure 21**\(^{35} \). Absorbance vs. concentration standard curve for the Fe\(^{2+}\)-Phenanthroline complex \((\lambda_{\text{max}} \sim 510 \text{ nm})\). The extinction coefficient is the slope of the line = 8.1 \( \times \) 10\(^{-3} \) M\(^{-1}\)cm\(^{-1}\).

### 2.3.1.2 ICP-AES [Fe\(^{2+}\)] Determination

Bare and surface-modified Fe\(_3\)O\(_y\) NPs were acid digested in the same manner as described in section 2.3.1.1 for the preparation of Fe(II)-o-phenanthroline samples; however, for ICP-AES analysis, 70% (15.7 M) of high-purity HNO\(_3\) (OmniTrace Ultra) was used instead of ACS grade HNO\(_3\). An aliquot of the acid-digested NP solution was diluted with nH\(_2\)O to create a 2% w/w HNO\(_3\) solution in a 15 mL conical centrifuge tube (minimum 3mL volume). Atomic emission intensities (Optima 7000 DV ICP-AES spectrometer) of Fe\(^{2+}\) in NP samples and in a series of Fe\(^{2+}\) standards\(^{11}\) (40 ppm, 4 ppm, 0.4 ppm, 0.04 ppm, 0.004 ppm) were obtained at \( \lambda_{\text{em}} = 238.204 \text{ nm} \). Output intensity values of standards were plotted as function of [Fe] in MS Excel to

\(^{11}\)Fe\(^{2+}\) standards were always run alongside NP samples. Therefore, a new standard curve was calculated for each batch of samples. This differs from the UV-Vis analysis in which [Fe] was calculated from a single standard curve shown in Figure 20.
create a standard curve and Beer’s Law (A = εcl) was then used to calculate [Fe^{2+}] in Fe_xO_y NP samples from sample intensity values.

2.3.2 MRI Phantom Preparation

Two methods for phantom preparation were explored. In the first method, agarose suspensions of Fe_xO_y NP samples in nH_2O were prepared using the procedure outlined by Yayla^5. In the second method, aqueous suspensions of Fe_xO_y NP samples in nH_2O were prepared as outlined below. An equal volume (0.5 – 1 mL) of 3-5 serial dilutions (typically 0.03 mM to 0.3 mM) of Fe_xO_y NP samples in nH_2O were pipetted into individual 2, 3, or 5 mm OD NMR tubes (Wilmad). In addition to a NMR tube containing pure nH_2O, sample NMR tubes were either a) suspended in 1.3 % w/v agarose gel doped with manganese in a conical 25 mL plastic centrifuge tube or b) put into a custom, reusable phantom holder designed by Stephanie Huang ’12 (Figure 22B).

![Figure 22](image_url)

Figure 22. (A) A T2-weighted cross-sectional MR image of eight 2mm NMR tubes suspended in a phantom holder (B). Tubes in the circle contain varying [SPIO – bare or functionalized with PEG and/or TEOS] in nH_2O; darker regions on MR image contain a greater [Fe]. The tube outside the circle contains pure nH_2O. Imaging Parameters: TR = 3062 ms, TE = 20 ms, FOV = 4.00 cm, 1 Avg, MSME-T2-MAP, Bruker ParaVision.

2.3.3 MRI T2 Relaxivity Studies

In order to obtain the T2 relaxation time of samples for molar relaxivity calculations, MR images (400 MHz Bruker Avance NMR spectrometer, 9.4T, vertical bore, 2.4 G/cm/A gradient strength) were acquired of the Fe_xO_y NP phantoms (section 2.3.2). MR Images were acquired with Bruker
Paravision 4.0 MSME-T2-MAP pulse sequence; optimized parameters were TR = 3062 s, TE = 10, 20, 30, 40, … , 600 ms, 60 echoes, 1 avg. 4 x 4 cm FOV, 128 x 128 matrix. An example MR image is shown in Figure 22A. T2 values were determined by fitting an exponential decay curve to a plot of TE vs. Signal Intensity for each [Fe₃O₇] using the Image Sequence Analysis (ISA) tool in Paravision as outlined in the Appendix of Yayla⁵. In MS Excel, molar relaxivity values were obtained by finding the slope of [Fe] (determined using UV-Vis or ICP-AES as outlined in section 2.3.1) vs. 1/T2 for each Fe₃O₇ dilution according to equation (8).
3 Results and Discussion

3.1 Surface Modification of Fe₃O₇ NPs

Commercially available SPIOs were coated with PEG and/or silica to 1) afford additional protection against oxidation of the iron oxide surface\(^{36}\) \textit{in vivo} and 2) facilitate future surface functionalization with anti-tumor moieties by introducing easily tunable surface chemistries.

Surface modification of the SPIO nanocores is currently required for clinical approval of SPIOs as MRI contrast agents. A thin coating can alleviate particle aggregation, afford greater biocompatibility, and, in the case of PEG, can increase the blood circulation time of SPIOs \textit{in vivo}\(^{37}\). For example, Feridex I.V. \textregistered, the first SPIO-based MRI contrast agent clinically approved for imaging liver lesions, is a dextran-coated colloidal suspension of SPIOs. The biocompatible carbohydrate coating effectively stabilizes agglomerates of SPIOs in aqueous dispersions, but its branched surface is prone to inter-particle cross-linking and it is therefore not a suitable scaffold for anchoring other functional groups. To serve as an effective chemical scaffold, a protective coating such as silica or PEG, with uniform and well-known surface chemistry, is desired.

3.1.1 Fe₃O₇@SiO₂ NPs

Hydrophilic silanol groups (-Si-OH) on the surface of silica can serve as versatile covalent and electrostatic anchor sites for various ligands. Silica-coated iron oxide NPs (SCIOs) have also shown low toxicity \textit{in vitro}, a promising biocompatibility consideration for their use as targeting and imaging agents \textit{in vivo}\(^{38}\). Silica is also net negatively charged at pH = 7, which contributes to anionic repulsive stabilizing forces in solution. Notably, the main difference between metallic gold – another scaffold-protective interface for SPIOs being investigated by Lisa Jacobs ‘12 at Wellesley College\(^{39}\) – and the “softer” polymeric matrix of silica is the nature of the chemical linker groups on the surface. The readily thiolated surface of gold exposes Au-thiol (-Au-SH)
groups whereas condensation of the silica exposes Si-hydroxyl (-Si-OH) groups. Both are well-understood and uniform surfaces that can covalently tether common functional groups like amines and carboxylic acids.

With these considerations in mind, a sol-gel procedure called the Stöber method was adapted from Ji et al. to coat SPIO cores (Ferrotec EMG 304®, 10 nm average size) with a thin layer (1-10 nm) of silica. In a sol-gel procedure, a colloid solution (sol) serves as a precursor for the formation of a polymeric matrix or distinct particles (gel). The source of silica in the Stöber method is a silicon alkoxide species (tetraethylorthosilicate – TEOS), which condenses onto the colloidal SPIO cores in a basic alcohol/water mixture to form the silica coating (Figure 23).

Figure 23. Ferrotec SPIO nanoparticles (EMG 304®) were coated with silica using the sol-gel process. A silica precursor, TEOS (tetraethylorthosilicate), replaces an unspecified anionic capping ligand on the SPIOs (not shown) and condenses onto the surface of the Fe₃O₄ nanoparticle (shaded sphere) under basic conditions. Hydroxyl groups on the surface of iron oxide react with the silica precursor and replace any non-covalent capping ligands to form a covalent Fe-O-Si bond. In many Stöber processes described in the literature, SPIOs are pre-synthesized with a weak capping ligand (most commonly oleic acid) to alleviate aggregation prior to and during silica coating. Although precise composition of the Ferrotec EMG 304® SPIO suspension is proprietary information, the MSDS indicates a
0.5-1.5% v/v anionic “water soluble dispersant” is present to stabilize SPIOs. If this “dispersant” stabilizes the SPIOs non-covalently, excess silica precursor in the synthesis should displace the dispersant to form the covalent Fe-O-Si bond. Notably, the relatively mild EtOH/water reaction conditions and ability to incorporate aqueous SPIO suspensions in a surfactant-free preparation make the Stöber process a more facile synthesis than organic-solvent based preparations.

### 3.1.1.1 [TEOS] affects monodispersity and size of SCIOs

The amount of TEOS (0.05-10% v/v) was varied in attempt to control the thickness of the silica layer in accordance with reports by Yayla and Deng et al. that the thickness of the silica coating can be controlled by adjusting concentration of the silicon alkoxide precursor. Because silica is visually more transparent than iron oxide under a TEM beam, TEM images of the NPs were obtained to assess the size and morphology of the desired core-shell SCIOs.

At the lowest concentration of TEOS (0.05% v/v), a thin (5-10 nm) silica coating was evident in the TEM image, however aggregation significantly obscured the particle morphology, resulting in a non-uniform core-shell structure and a wide particle size distribution (Figure 24).

![Figure 24. TEM images of core-shell silica coated Ferrotec SPIOs reported by Ji et al. (A) and in this study (B) using a sol-gel process (V_EW = 5; 28% wt. NH₄OH 8.75% v/v; 12 hour reaction time) with 0.5% v/v TEOS (A) and](image-url)
0.05% v/v TEOS (B). In (A) there is a relatively uniform transparent layer of silica around darker iron oxide cores (indicated by the arrows). In (B) the core-shell structure is only evident in some regions (one highlighted by the white box inset); significant aggregation and a wide size distribution is also evident.

Figure 24 also highlights another interesting phenomenon. Using nearly identical sol-gel reaction conditions, Ji et al.\(^7\) obtained more monodisperse SCIOs at a higher [TEOS] (Figure 24A) than used in this study (Figure 24B). In their silica sol-gel characterization studies, Deng et al.\(^{33}\) also reported more monodisperse and spherical SCIOs at high [TEOS] relative to low [TEOS]. One plausible explanation for these differences may be that at lower [TEOS], the silanol-SPIO interaction is not sufficient to overcome inter-particle SPIO interactions arising from the unspecified capping agent. As the [TEOS] is increased, however, Le Chatelier’s principle favors the silanol-SPIO interaction, which results in more well-defined core-shell structures. The observed trade-off between particle size and monodispersity motivated alternative modifications to the Stöber method to selectively tune the size of the silica coating without compromising uniform size distribution of particles.

### 3.1.1.2 Solvent composition influences SCIO particle morphology

Deng et al.\(^{33}\) reports that the concentration of any reagent in the sol-gel process (i.e. TEOS, NH\(_4\)OH, EtOH, and/or H\(_2\)O) can be altered to fine-tune particle dispersion and morphology. In the context of the sol-gel reaction conditions used in this study, the most compelling result from the Deng et al. study was the observation of irregular SCIO particle morphology when the volume ratio of ethanol to water (V\(_{E:W}\)) was lower than 2 or higher than 4. Given that the V\(_{E:W}\) adapted from the Ji et al. protocol\(^7\) is outside of this range (V\(_{E:W} = 5\)), the next logical step was to investigate the effect of V\(_{E:W}\) on particle morphology and dispersion.
Figure 25. TEM images of silica-coated Ferrotec; EtOH/H$_2$O ($V_{EW} = 2$-6, TEOS 0.05% v/v, 28% wt. NH$_4$OH 8.75% v/v; 12 hour reaction time).

Figure 25 shows representative TEM images of SCIOs synthesized with various $V_{EW}$ ranging from 2-6. While differences in morphology are visually evident among the different $V_{EW}$, it is clear from the aggregation and non-uniform particle size in all three cases that the problem of monodispersity is still unresolved. Notably, however, $V_{EW} = 4$ provides the best of the three particle morphologies. Aggregation appears to be most problematic in $V_{EW} = 6$ and unreacted silica – a phenomenon Deng et al. term “phase separation” – appears to dominate in $V_{EW} = 2$. This result can be rationalized by the fact that SPIOs are more stable in the water than EtOH while the silica precursor, TEOS, dissolves more readily in EtOH. Thus, if there is too much water (low $V_{EW}$ value), silica may undergo a phase separation and condense onto itself in the EtOH as it appears to have done in $V_{EW} = 2$. On the other hand, if there is too little water (high $V_{EW}$ value), irregular particle size and morphology may result because SPIOs are not dispersed efficiently in the solvent$^{33}$. In $V_{EW} = 4$ there is evidence of core-shell structure without phase separation or excessive aggregation. However, the SCIOs lack spherical and particulate morphology; instead they are associated with a matrix of silica. Given that a low [TEOS] was used in this reaction (0.05% v/v), this matrix effect may be due to the same trade-off between overall SCIO size and morphology observed when varying [TEOS]. In order to investigate this further, the same $V_{EW} = 4$ reaction conditions were repeated with a higher (0.5% v/v) and lower (0.009% v/v) [TEOS]s. TEM images of SCIOs from each [TEOS] are shown in Figure 26.
Figure 26. TEM images of silica-coated Ferrotec using (A) 0.009% TEOS and (B) 0.5% TEOS; E/W = 4, 28% wt. NH$_4$OH 6.25-8.75% v/v. SPIO cores are visible as dark dots (denoted by arrow) coated by a more transparent silica layer.

Indeed, a more globular and particular core-shell structure is observed at the higher [TEOS] than the lower [TEOS], suggesting the size-monodispersity trade-off is a persistent limitation of the Stöber process. One note that should be made of this analysis and TEM analysis as a whole is that it is difficult at times to differentiate between SPIO cores and the silica layer, especially at a low [TEOS] (Figure 26A). This may result from not using enough TEOS to see any appreciable silica coating and/or poor TEM resolution of the SPIO crystal lattice (Nolan T. Flynn, Personal Communication). It is plausible, for example, that the iron oxide crystal lattice is oriented in different ways relative to the electron beam path of the TE microscope, which may result in different intensities of “darkness” that are evident in TEM images of SPIO cores. Despite instrumental limitations, however, there was ambiguous and poor evidence from TEM even at moderate [TEOS] that the sol-gel process was yielding a monodisperse and thin layer of silica on the SPIO cores.

3.1.1.3 PEG affects DLS (but not TEM) SCIO particle size in microemulsion

In attempt to overcome limitations of the sol-gel process, SPIO cores were also coated with silica using a water-in-oil inverse microemulsion process. Narita et al. underscore that while the sol-gel process has the advantage of being a relatively simple reaction procedure, it occurs on
the timescale of several hours and is too fast to offer efficient size control. On the other hand, a slightly more involved microemulsion procedure can offer better morphological control by using a non-ionic surfactant to mediate the silica coating around iron oxide cores in reverse micelles. A reverse emulsion phase (Figure 27) was established by replacing the H₂O/EtOH solvent in the Stöber method with an organic solvent (cyclohexane) – the “oil” component – and a non-ionic surfactant (Igepal CO-520) to dissolve the “water” phase NPs. Given the reported success of the microemulsion process in synthesizing small monodisperse silica-coated metal NPs (< 20 nm), this was investigated as a promising synthetic alternative.

![Figure 27](image)

**Figure 27**. Reverse microemulsion synthetic scheme for Fe₃O₄@SiO₂ NPs in cyclohexane.

During the initial microemulsion synthesis, the TEM was under repair and SCIOs were analyzed with DLS and FT-IR spectroscopy. DLS measurements, which give the hydrodynamic radius of small particles in solution, have proven difficult for SPIOs because despite the “water soluble dispersant,” they readily aggregate in solution, especially at higher concentrations. DLS measurements for bare SPIOs, for example, were only successfully obtained at or below 4 x 10⁻³ mg/mL of the Ferrotec suspension. Occasionally DLS measurements were not consistent and/or did not meet quality criteria required by the analysis software. Therefore, DLS data should be considered preliminary.
Figure 28. DLS measurements of overall SCIO size after (A) Microemulsion synthesis and (B) Post-microemulsion addition of PEG-Silane (MW = 420); note that values shown are “calculated” and further averaging should be done to obtain a standard deviation to more accurately report values.

The initial SCIO particle diameter reported for the microemulsion synthesis by DLS was 107 nm. Given the DLS diameter of uncoated SPIO cores is ~ 20 nm, a ~100 nm particle size indicates a ~ 40 nm corona of silica around the SPIO core (Figure 28A). Though it is reasonable to expect the hydrodynamic diameter of particles in solution to be larger than size measurements of dry samples obtained from TEM images, a 40 nm increase far exceeds the increase predicted by the formation of a thin (5-10 nm) coat of silica, suggesting the particles might be aggregating in solution. To probe this further, a 10 μL aliquot of PEG-silane (MW = 420) was sonicated and stirred with the post-reaction mixture. Because PEG lacks the reactive alkoxide terminal groups on TEOS, it was expected to alleviate inter-particle aggregation resulting from potential cross-linking of TEOS. The 92 nm DLS diameter of the PEGylated SCIOs is expectedly smaller than the unPEGylated SCIOs (107 nm), however this 15 nm decrease is not significant enough to verify the deposit of < 20 nm silica layer (Figure 28B).

Moreover, PEG’s hydrophilicity may also increase the hydrodynamic diameter of the particle and therefore the observed decrease in particle diameter post-PEGylation might be less than expected if another, less hydrophilic, stabilizing agent been added to relieve aggregation. That is, SCIO particle diameter post-PEGylation cannot be directly correlated with thickness of
deposited silica layer because size increase due solely to PEGylation of the SPIO surface could not be taken into account.

Nonetheless, PEG’s reported ability to prolong the half-life of drugs in the body\(^9\) and its potential to serve as an Avidin-biotin linker for additional functionalization of the nanovehicle through a modified biotinylated-PEG species motivated further studies with the microemulsion silica synthesis. Park et al. were successful, for example, in depositing a thin (4.5 ± 1.0 nm) layer of silica on SPIO cores in the microemulsion procedure by adding a long-chain alkane silane that lacks the reactive alkoxide groups on TEOS\(^{44}\). One reasonable interpretation of adding the alkane silane is that the unreactive alkane terminus limits the amount of silica that can condense on the NP surface and alleviates inter-particle condensation and aggregation. By the same logic, a PEG-silane species was added during or after microemulsion synthesis to see if similar results could be obtained.

Due to poor S:N, the FT-IR spectrum in Figure 29 suggests, but does not confirm, the presence of a silica-PEG interface on the SPIO cores when PEG-silane (MW = 420) was added with TEOS during the microemulsion synthesis.

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**Figure 29.** FT-IR spectra of Fe\(_2\)O\(_3\)@SiO\(_2\) NPs and Fe\(_2\)O\(_3\)@(SiO\(_2\)+PEG) NPs synthesized using the microemulsion method. Trimethoxysilane PEG (MW = 420) was added to PEGylated sample at the start of reaction with TEOS. Samples were washed/collection in EtOH and left to dry for 5-10 minutes on sample holder before IR spectra were obtained. Note appearance of (noisy) C-H stretch (2927 cm\(^{-1}\)) and shifted Si-O stretch (1082 cm\(^{-1}\)) in PEGylated sample.
In Figure 29 there is (noisy) evidence of PEG sp³ C-H stretches that did not appear to be in the IR spectrum for the microemulsion silica synthesis without PEG. Moreover, the Si-O stretch in the PEGylated sample (1082 cm⁻¹) is shifted slightly from the Si-O stretch from TEOS in the unPEGylated sample (1059 cm⁻¹), which may also indicate the presence of the silane group from the PEG. On the other hand, FT-IR spectra did not reveal any change in C-H or Si-O stretch regions when PEG was added after the microemulsion synthesis with the PEG/EtOH/nH₂O terminating solution (Figure 30).

![Figure 30. FT-IR spectra of Fe₃O₄@SiO₂ NPs synthesized using the microemulsion method and terminated with either EtOH or a PEG/nH₂O/EtOH solution. Samples were dissolved in water and run with a water background scan. Note that no changes in vibrations are seen in C-H in Si-O stretch regions.](image)

Notably, it is hypothesized that the presence of water and its insolubility in the organic reaction media segregated the PEG from the SPIO cores to not allow sufficient (or any) PEG adsorption detectable by FT-IR. Post-reaction surface modification with PEG was repeated without water using a PEG/EtOH terminating step, but sample was too dilute to obtain readable IR spectra of this product. On an important side note, the silane (Si-O) stretches appear “sharper” and more resolved in Figure 30 than in Figure 29. Differences in appearance of the stretch may be due to

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[12] Another reason for the poor S:N and a notable complication of interpreting the spectra in Figure 29 is the relative amount of sample measured in each case. Because the PEGylated sample may have been more dilute, FT-IR data should be re-obtained to confirm PEGylation. Additionally, slight differences in the two spectra could also be attributed to an unequal amount of time given for the samples to “dry” on the ATR crystal (thus, the possibility of seeing a signal from the solvent, EtOH) and this should be investigated further.
one or more factors: the phase of the sample (liquid vs. dry), number of averages, and different
resolutions ($\text{cm}^{-1}$) used to collect spectra. Future work should investigate optimum number of
averages and resolution to achieve high-quality, resolved FT-IR spectra.

TEM analysis was performed on reactions 1) with PEG added during the reaction, 2) terminated with PEG/EtOH, and 3) terminated with EtOH only.

Upon visual inspection, there was no apparent difference in particle size and morphology among samples with different volume ratios of PEG to TEOS ($V_{P:T}$) added during the reaction in TEM images (Figure 31).

![Figure 31. TEM images of Fe$_x$O$_y$@($\text{SiO}_2 + \text{PEG}$) NPs synthesized using the reverse microemulsion method terminated with EtOH after ~ 24 hours with $V_{P:T}$ (PEG to TEOS volume ratio) = 2-8.]

PEG in the reaction media was hypothesized to limit aggregation and the amount of TEOS that condenses onto the surface of the SPIO, but $V_{P:T} = 2, 4, \text{ and } 8$ all have similar particle sizes and ambiguous core-shell morphologies. Furthermore, although overall particle size is < 20 nm; it is not obviously apparent that this is “better” than the Stöber method in which similar aggregation was evident in TEM images. Because the silica coating is not uniform and distinct SPIO cores are difficult to visualize given considerable aggregation, evidence of PEGylation merits further investigation. Moreover, given the indirect FT-IR evidence of PEG on the surface and different molar relaxivity values (section 3.2.2) for the samples with different $V_{P:T}$ values shown in Figure 31, there is evidence that surface chemistry of SCIOs is changing – it just may not be able to be detected using TEM.
TEM analysis also revealed no major qualitative differences between products terminated with PEG/EtOH or EtOH only. It was expected that SCIOs terminated with PEG/EtOH would be less aggregated and/or smaller than SCIOs terminated with only EtOH owing to the presence of PEG, but as TEM images shown in Figure 32 reveal, particle morphologies are nearly identical in both cases. This, notably, contradicts DLS evidence discussed above that overall particle size changed when PEG was added to SCIOs post-synthesis. However, given PEG’s hydrophilicity, DLS may be more sensitive to changes in particle diameter than TEM.

![Image of TEM images](image)

**Figure 32.** TEM images of Fe$_3$O$_4$@SiO$_2$ NPs synthesized using the reverse microemulsion method terminated after 1 hour with A) PEG/EtOH and B) EtOH

Ultimately, although TEM offers insight into core-shell morphology, it was difficult to glean evidence of PEG surface functionalization during or after microemulsion synthesis from TEM images. Notably, manual quantitative measurements of particles using the current TEM instrument are also approximate and a statistical comparison would require a tedious random sampling over a large area. The low resolution (~ 0.5 nm) of the TEM instrument is a significant limitation of any quantitative investigation. Thus, DLS may be a better characterization method to assess and tune PEG functionalization if aggregation limitations can be overcome.

### 3.1.1.4 Microemulsion reaction time can tune SCIO particle size

Simultaneous to investigating the effect of PEG on SCIO particle size and morphology, TEM was also used to see if the size of the silica layer could be tuned by varying reaction time. This study was motivated by Narita et al. who reported that thickness of the silica layer could be
tuned to 2-10 nm by using 1-10 hour reaction times, respectively\textsuperscript{34}. To investigate this further, TEM images were taken of the PEG/EtOH terminated product of the microemulsion silica synthesis after 1, 2, 3, and 5 hours (Figure 33).

![TEM images of Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2} NPs synthesized using the reverse microemulsion method terminated after 1, 2, 3, and 5 hours with PEG/EtOH. To emphasize particle morphologies, a yellow border was drawn to denote silica boundaries and a white border was drawn to indicate the Fe\textsubscript{3}O\textsubscript{4} core of selected particles.](image)

Using the crude TEM measurement protocol, a 1-5 nm layer of silica was evident around SPIO cores for all time points, but no consistent patterned increase in size was observed. The most monodisperse particles were observed at the 1 hr reaction time, which had consistent 1-3 nm thick silica coatings and visibly particulate character (as opposed to being caught in a matrix of silica as shown in the 2 hr and 5 hr time points in Figure 33). Polydispersity remained a critical issue of the silica coating process for the 2, 3, and 5 hr batches. Often, aggregates and/or various sizes of NPs were evident in nearby clusters again; Figure 34 is a representative TEM image of this phenomenon.
Figure 34. TEM image of polydisperse Fe$_{x}$O$_{y}$@SiO$_{2}$ NPs synthesized using the reverse microemulsion method terminated after 3 hours with PEG/EtOH.

The evident core-shell morphology and thin (1-3 nm) silica coating of the 1 hr batch is the most promising synthetic outcome of silica syntheses explored to date for the purpose of building a versatile platform for targeted cancer therapy (Figure 35).

Figure 35. TEM images of Fe$_{x}$O$_{y}$@SiO$_{2}$ NPs synthesized using the reverse microemulsion method terminated after 1 hour with PEG/EtOH. Images are identical, but shown at different contrast/brightness levels to emphasize silica coating (boundary drawn around one NP in yellow on the left image) and iron oxide cores (boundary drawn around one NP in white on right image).

One drawback, however, was the observation of large clumps (> 50 nm) of silica in certain regions of the TEM grid. Theoretically, collection by a magnet should be sufficient to isolate only the Fe$_{x}$O$_{y}$@SiO$_{2}$ particles, but Narita et al. also propose additional centrifugation steps that should be explored to eliminate the excess silica$^{34}$. It is difficult to propose a trend for the nature of aggregation taking place at the other time points because TEM is not an ensemble measurement like DLS, for example. Notably, however, less aggregation for the shortest time point (1 hr) overcomes the size-monodispersity trade-off observed in the Stöber method, which favored the particulate nature of larger particles. This is likely due to the role of the surfactant
around the SPIOs, which selectively controls how and how much of reagents can interact with the SPIO surface. In this regard it is plausible that at longer time points more TEOS may be able to get “inside” the micelle formed by the non-ionic surfactant, thereby disrupting the micelle structure and facilitating the formation of aggregated and less monodisperse matrices of SPIOs embedded in silica seen at 2 hr and 5 hr time points in Figure 33.

3.1.2 Fe₅O₇@PEG NPs

An alternative scaffold-protective layer for the SPIO core bypasses dense polymeric and metallic matrices (e.g. silica and gold, respectively) altogether and involves functionalizing the SPIO surface with a hydrophilic and biocompatible ligand such as PEG. Terminal hydrophilic hydroxyl groups on PEG 1) afford antifouling properties that can enhance blood half-life of a PEGylated species in vivo and 2) serve as versatile attachment sites for functionalization with common chemical functional groups and biomolecules, such as biotin. An early concern with PEG functionalization on SPIO surfaces was that it often took place via noncovalent dipole-dipole interactions that would be unstable in biological environments. However, Lee et al. demonstrated that SPIOs coated with a PEG-silane species are indeed stable and have MRI-contrast agent capability in vivo, which can be attributed in part to the strong silane anchoring group that covalently attaches the PEG species to the SPIO surface.

3.1.2.1 FT-IR and TEM confirm PEGylation of SPIOs in EtOH

The FT-IR spectrum of the PEGylated product reveals the presence of sp³ C-H stretches and a fingerprint region with a prominent C-O bend associated with the polymeric backbone of the PEG-silane species (Figure 36). Although FT-IR confirms the presence of PEG, it does not give an indication of whether or not it is adsorbed to the SPIO surface. Despite the wash and collection steps after PEGylation that attempt to get rid of unreacted PEG in solution, ineffective
wash steps could allow freely floating PEG to contribute to the IR signal. Therefore, to confirm efficacy of these washing steps, a negative control functionalization of SPIOs was performed with a PEG-thiol species (MW = 2300). Because thiol (-SH) groups lack covalent affinity to the hydroxyl groups on the SPIO surface, no PEGylation was expected to occur and, thus, any signal from PEG in the IR spectrum of the product post-washing can be attributed to freely floating PEG and indicate poor efficacy of washing steps. No PEG signature is seen in the IR spectrum of the PEG-thiol functionalization, which is an indirect confirmation that PEG is adsorbed to the surface in the PEG-silane functionalization.

![Figure 36](image-url) FT-IR spectra of Trimethoxy PEG-silane (MW = 1050), Bare Fe₃O₄ (EMG 304®), Fe₃O₄@PEG-thiol (MW = 2300), and Fe₃O₄@PEG-silane (MW = 1050) NPs. Functionalization with PEG species was performed in EtOH ~ 24 hours. Samples were washed/colllected in EtOH and left to dry for 5-10 minutes on sample holder before IR spectra were obtained. Note presence of C-H stretch and PEG fingerprint vibrations in PEG-silane, but not PEG-thiol Fe₃O₄@ functionalization.

TEM images of SPIO cores with and without PEG also gave additional visual confirmation of successful PEGylation (Figure 37). The increased inter-particle distance among PEGylated SPIO cores (vs. bare SPIO cores) is congruent with the attachment of PEG to the surface of SPIOs.
Figure 37. TEM images of A) bare and B) PEGylated SPIO cores. Visually there is an expected increase in inter-particle distance of the PEGylated product (indicated in selected regions by yellow arrows) as compared to the bare SPIO cores, which appear more aggregated.

### 3.2 MRI Contrast effect of Fe$_x$O$_y$ NPs

To quantify the MRI contrast capabilities of SPIOs, a protocol for measuring T2 molar relaxivity ($r_2$) of SPIOs was first optimized building on previous work (section 3.2.1) and then $r_2$ values were calculated for surface-modified SPIOs (section 3.2.2) to understand the impact of surface chemistry and overall particle size on improving, maintaining, or worsening T2 contrast of SPIOs in MR images.

#### 3.2.1 Different $r_2$ values in H$_2$O vs. Agarose

T2 relaxivity studies previously performed on SPIOs by Yayla$^5$ used phantoms that contained tubes of SPIOs suspended in agarose and yielded $r_2$ values ten times greater than $r_2$ values reported in literature (see Table 2 in Introduction). This discrepancy is likely attributed to the fact that it is standard practice to report molar relaxivity values for SPIOs dissolved in water instead of agarose. Repeating the T2 relaxivity study outlined by Yayla on bare SPIOs dissolved in water did, in fact, yield a $r_2$ value (470 ± 80$^{[13]}$ mM$^{-1}$ s$^{-1}$; n = 3) a factor of ten times smaller.

$^{[13]}$ The standard deviation calculated for n = 3 independent measurements of molar relaxivity may be associated with a variety of factors: uncertainty associated with concentration measurements, dilutions, and/or slight variations in automated scan parameters for each imaging session. With regard to the latter, efforts were made to alleviate such
than the molar relaxivity value originally obtained with agarose (4000 mM$^{-1}$ s$^{-1}$)$^5$, confirming this hypothesis. It is expected that absolute T2 relaxation times should be different in water and agarose because agarose - a gel - is a more “compact” environment that will facilitate more spin-spin dephasing than pure water, resulting in a smaller T2 time for agarose. Because $r_2$ is inversely related to T2 according to equation (8), a smaller T2 value should yield a higher $r_2$ value for agarose, which is indeed what was observed. Interestingly, Parkes et al. reported $r_2$ values that were independent of agarose or water suspensions of cobalt NPs$^{46}$. Parkes claims this indicates there is no further aggregation of particles upon dispersion in agarose. However, it may also be possible that aggregation is offsetting the effect of the medium – agarose or water, as discussed above – on $r_2$. If the latter is occurring, our results suggest particles are aggregating differently in agarose than water since there is a ten-fold difference in molar relaxivity values. Specifically, if deviation from literature $r_2$ values is a marker of aggregation, it suggests our particles are, in accordance with Parkes’ implication, aggregating more in agarose than water.

Whatever the cause of the discrepancy (i.e. suspending medium and/or aggregation), water suspensions of SPIOs proved much easier to handle and prepare than the agarose counterparts. Most importantly, the $r_2$ value (470 mM$^{-1}$s$^{-1}$) is consistent with those reported in the literature. Ji et al. report, for example, a $r_2$ value of 162 mM$^{-1}$s$^{-1}$ for the same Ferrotec EMG 304® SPIO cores used in this study. Although this is lower than the value obtained in our study, the discrepancy in this case can be attributed to the different methods used to determine [Fe].

Not unexpectedly, the 470 mM$^{-1}$s$^{-1}$ $r_2$ value for bare SPIO cores is higher than the range (50 – 150 mM$^{-1}$s$^{-1}$) of $r_2$ values reported for polymer-coated SPIO contrast agents reported in Table 2. As discussed in the introduction, size and surface chemistry of polymer coatings can increase and/or decrease $r_2$. Ideally, we want to maximize $r_2$ (higher $r_2$, smaller T2, better contrast) so that variations by imaging all sample concentrations in a single scan by using the phantom holder designed by Stephanie Huang ’12 (Figure 22), which can accommodate up to seven 3 mm NMR tubes.
it is within or above the range of $r_2$ values in Table 2. In this regard, having a high $r_2$ for bare SPIO cores is promising because it offers some room for acceptable loss in contrast due to, for example, the physical increase in size that accompanies deposition of a layer or PEG or silica onto the SPIO surface.

### 3.2.2 $r_2$ values of Fe$_x$O$_y$@X NPs a function of size and surface chemistry

In all trials for surface modification of SPIOS with PEG and silica, the $r_2$ value decreased to differing degrees (Table 6) from the bare SPIO $r_2$ value ($470 \text{ mM}^{-1}\text{s}^{-1}$). This makes sense: regardless of contrast-enhancing surface chemistry interactions that can improve $r_2$ by “pulling” more water toward the SPIO core, functionalization procedures nonetheless contribute to an overall size increase of the NP that will tend to distance water molecules from the SPIO core, lessening the degree of spin-spin relaxation and resulting in lower $r_2$ values (decreased contrast).

<table>
<thead>
<tr>
<th>Sample</th>
<th>$r_2$ ($\text{mM}^{-1}\text{s}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$_x$O$_y$ – Ferrotec EMG 304®</td>
<td>470 ± 80 (N = 3)</td>
</tr>
<tr>
<td>@ PEG (MW = 2300) (Trimethoxysilane PEG)</td>
<td>365</td>
</tr>
<tr>
<td>@ PEG (MW = 420) (Trimethoxysilane PEG)</td>
<td>99</td>
</tr>
<tr>
<td>@ PEG (MW = 420) + TEOS</td>
<td>31</td>
</tr>
<tr>
<td>@ APTMS + 2 nm Au NPs</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 6. Calculated $r_2$ values for bare and Fe$_x$O$_y$@X functionalized NPs

Notably data from Table 6 reveal that modification with PEG, a hydrophilic species, shows the smallest decrease in $r_2$ from bare SPIOs$^{[14]}$. In fact, although the relaxivity of PEGylated NPs is lower ($365 \text{ mM}^{-1}\text{s}^{-1}$ and $99 \text{ mM}^{-1}\text{s}^{-1}$) than bare SPIOs, it is still within or above the range of $r_2$ values for clinical MRI contrast agents (Table 2). Given the various synthetic alternatives in construction of the nanoparticle platform for targeted cancer therapy, this result suggests that

$^{[14]}$ It is counterintuitive for the 2300 MW PEG to have a higher $r_2$ ($365 \text{ mM}^{-1}\text{s}^{-1}$) than the 420 MW PEG ($99 \text{ mM}^{-1}\text{s}^{-1}$), but this may be due to the different $[\text{PEG}]$ in each functionalization procedure.
PEG may be a favorable component of the scaffold-protective layer around SPIOs not only to improve biocompatibility, but to enhance MR image contrast as well. Moreover, LaConte et al. report that increasing the PEG coating thickness around 6.6 nm SPIO cores decreases $r_2$, but PEG coatings of $5000 > \text{MW} > 750$ have similar relaxivity values$^{36}$, suggesting at a certain size along the functionalization process surface chemistry may play a larger role than an increase in size associated with functionalization in affecting $r_2$. This implies that PEG and other biological species that associate with water (e.g. Avidin, biotin, Poly-GL) on the surface of the nanovehicle may also have the ability to cause a net$^{[15]}$ increase in $r_2$ during later stages of the nanoparticle assembly process. Given the low $r_2$ value measured for Au-seeded SPIO cores (synthesized by Lisa Jacob ’12), the surface chemistry of moieties at the surface of the NP will play an especially important role in maintaining and/or improving MR contrast.

Although time did not permit calculation of $r_2$ for Au-coated SPIOs, intensity analysis was performed on a MR image of a phantom containing dilute concentrations of 11.2 Au NPs, Fe$_x$O$_y$@Au NPs, and Fe$_x$O$_y$@(APTMS + 2nm Au NP seeds) (samples synthesized by Lisa Jacob ’12). The MR phantom image and corresponding absolute T2 relaxation curves (TE vs. signal intensity) are shown in Figure 38. Although absolute T2 relaxivities cannot be used to compare the relative efficacy of Au-modified SPIOs to each other or the other surface modified NPs listed in Table 6 (since the [Fe] is not taken into account), it is useful to note that Fe$_x$O$_y$@Au NPs and Fe$_x$O$_y$@(APTMS, 3-Aminopropyltrimethoxysilane, + 2nm Au NP seeds) do produce detectable negative contrast in the MR image and have faster T2 relaxation times than water and Au NPs according to the relaxivity curves. Au NPs serve as a control because they lack SPIO and do, as expected, have a relaxivity curve that matches that of pure water. These preliminary results

$^{[15]}$Net refers to taking into the account NP size increase and surface chemistry modification that contribute to overall $r_2$
indicate Au does not visibly inhibit the MR effect of SPIOs and \( r_2 \) values should be obtained to quantify this further.

**Figure 38.** (A) MR image of a phantom containing tubes with nanopure water \((nH_2O)\), Ferrotec@Au \((@Au)\), Ferrotec@(APTMS + 2nm Au NP seeds) \((@2nmAu)\), and 11.2 nm Au NPs \((Au \, NPs)\). Corresponding absolute relaxation curves are shown in (B). Note that 11.2 Au NPs have a similar absolute relaxation curve to water while samples containing Ferrotec have faster relaxation curves, indicating a MR-contrast effect, which is visible in the MR image for @Au.

In addition to calculating \( r_2 \) values for single batches of modified SPIOs reported in Table 6, \( r_2 \) values were also calculated for various surface modification reaction conditions to serve as another characterization tool to understand the MR contrast effect of coating SPIOs with various amounts of silica and/or PEG. In other words, different \( r_2 \) values for different reaction conditions can be indirectly attributed to a change in surface chemistry and/or size of NPs and vice versa.

\( r_2 \) values for SPIOs with 1) varying [PEG]s added during the microemulsion synthesis and 2) different reaction times in the silica microemulsion synthesis are shown in Figure 39 and Figure 40, respectively. As expected, \( r_2 \) values increase with an increasing [PEG]. This is rationalized by PEG’s hydrophilic, oxygen rich backbone that can retain water in the proximity of the SPIOs for enhanced relaxation. Notably, however, as discussed in section 3.1.1.3, it is not clear if the PEG is actually adsorbed to the SPIO surface; changes in \( r_2 \) could be attributed to residual PEG in the solution. Thus, it might be useful to perform a control study analogous to that performed for the PEGylation wash and collection steps discussed in section 3.1.2.1 to confirm PEGylation on particle surface.
Figure 39. T2 molar relaxivity \( (r_2) \) values were calculated for Ferrotec samples with different PEG/TEOS ratios added during the reverse microemulsion silica synthesis (~24 hour reaction time, terminated with EtOH; TEM images of NPs shown in Figure 31).

Despite the fact no significant changes in overall particle size at different time points of the silica microemulsion synthesis were detected using TEM (section 3.1.1.4), there is a decrease in the \( r_2 \) of Fe\(_x\)O\(_y\)@SiO\(_2\) as reaction time increases (Figure 40).

Figure 40. T2 molar relaxivity \( (r_2) \) values were calculated for Ferrotec samples at different time points in the reverse microemulsion synthesis terminated with PEG/nH\(_2\)O/EtOH solution.

This is most readily rationalized by an increase in particle size expected at longer time points in the microemulsion synthesis, but this was not consistently observed qualitatively in TEM images (section 3.1.1.4). Of course, other factors such as aggregation and clustering of NPs (evident, for example, at longer time points in TEM images) may influence relaxivity as well\(^7\). Ultimately,
changing $r_2$ values suggest the silica coating is being altered in some way around the SPIO core as a function of reaction time. To support and investigate this conclusion further, DLS may be able to quantify the corresponding changes in SCIO particle size and/or aggregation that were ambiguous in TEM. A particularly notable parallel between TEM and $r_2$ data, however, is the rapid decrease in $r_2$ from the 1 hr to 3 hr time points not seen between 3 hr to 5 hr. This supports the TEM image observation that SCIOs at 1 hr (1-3 nm silica thickness, largely monodisperse) were decidedly distinguishable from those at later time points, all of which appeared to suffer from similar degrees of polydispersity and SPIO-silica matrices. That is, less variation in $r_2$ between the 3 hr and 5 hr than the 1 hr and 3/5 hr time points supports the conclusion from TEM that morphology and/or particle size is not changing as significantly between 3-5 hours. With regard to producing monodisperse, small, and sensitive MRI-active NPs, therefore, TEM and $r_2$ data both suggest the 1 hr microemulsion synthesis is ideal.
4 Conclusions and Suggestions for Future Work

This study investigated a variety of synthetic routes and characterization methods to build a small, monodisperse, surface-modified SPIO core that can serve as a platform for building a multifunctional cancer therapeutic-diagnostic nanovehicle. There were three main areas of investigation:

1. Fe₃O₅@SiO₂ NPs were synthesized using Stöber and reverse microemulsion methods. A size-monodispersity trade-off was observed for the Stöber process, which precluded the synthesis by this method of small, monodisperse NPs. Small (15-20 nm overall size, 1-3 nm silica layer thickness), monodisperse Fe₃O₅@SiO₂ NPs were ultimately synthesized with the reverse microemulsion method using a short reaction time (1 hr), but NPs were observed using TEM to have small (0-1 nm) inter-particle distances (i.e. heavily aggregated). A PEG-silane species was added during and after microemulsion syntheses to try and alleviate aggregation, but deposition of PEG could not be confirmed by TEM. DLS, FT-IR spectroscopy, and molar relaxivity studies suggest addition of PEG is changing the surface chemistry of Fe₃O₅@SiO₂ NPs, but these results, especially DLS (preliminary) and FT-IR (poor S:N), need to be investigated further to confirm the synthesis of Fe₃O₅@SiO₂@PEG NPs.

2. Fe₃O₅@PEG-Silane NPs were successfully functionalized (confirmed using FT-IR and TEM). The next step is to functionalize Fe₃O₅@PEG-Silane NPs with biotinylated-PEG to take advantage of the affinity between Avidin-biotin proteins, which can serve as the “glue” for attaching anti-tumor and targeting agents to the NP surface.

3. T2 molar relaxivity studies were successfully conducted on bare (Ferrotec EMG 304®) and surface-modified SPIOs to quantify contrast produced in MR images and to serve as another characterization method to monitor changes in surface chemistry and size of the
Fe$_x$O$_y$@PEG and Fe$_x$O$_y$@SiO$_2$ synthesized NPs. All surface-modified SPIOs had lower $r_2$ values than bare SPIOs (~400 mM$^{-1}$s$^{-1}$). PEGylated species had the least deviation from bare $r_2$ values, likely owing to the favorable hydrophilic surface chemistry that offsets the unfavorable addition in size-increase of the particle. A preliminary absolute T2 relaxation phantom study also confirmed that Fe$_x$O$_y$@Au NPs had detectable MR negative contrast. The next step is to calculate $r_2$ values for Fe$_x$O$_y$@Au NPs and surface-modified SPIOs at each stage of the synthetic process. Ideally, $r_2$ values should be at or above ~100 mM$^{-1}$s$^{-1}$, which is the average T2 molar relaxivity of clinical SPIO-based contrast agents.$^{18}$

The main area for future improvement is in the synthesis of Fe$_x$O$_y$@SiO$_2$ NPs. Aggregation of Fe$_x$O$_y$@SiO$_2$ NPs was a key issue in all synthetic routes and there was a lack of quantitative characterization methods to monitor changes in size and surface chemistry of NPs.

There could be several culprits for the observed aggregation in silica syntheses. Roca et al. propose the morphology of silica-coated nanostructures is a product of competing chemical interactions among silica, the SPIO surface, capping ligands, dipolar forces between SPIOs that cause aggregation, and the stability of the protective coating itself.$^{47}$ Several studies, which have, in turn, guided our research, have already investigated some of these parameters by reporting the influence of water/alcohol ratio, the concentration of TEOS, and overall reaction time on the final morphology of the Fe$_x$O$_y$@SiO$_2$ NPs.$^7,33$ Yet, results from our study suggest aggregation persists in all permutations of the silica synthesis. A recent analysis on the role of SPIO capping ligands, however, demonstrated that ligand functional groups have a remarkable influence on the aggregation of SPIOs encapsulated by silica using the Stöber procedure.$^{47}$ Specifically, Roca et al. reported that SPIOs coated with tri(ethylene glycol) (TREG) favored the formation of silica aggregates while SPIOs coated with dimercaptosuccinic acid (DMSA) showed excellent long-term, individual particle stability. Carboxylate and thiol moieties on DMSA were hypothesized
to prevent SPIO aggregation by affording added stability to DMSA-coated SPIOs in solution.

Given there is an unknown “water-soluble dispersant” in the commercially-synthesized Ferrotec EMG 304® SPIO colloidal suspension, it may be worth investigating in SPIOs with different capping ligands to see if/how this affects NP aggregation in silica syntheses.

Alternatively, Narita et al. also report that aggregation of core-shell Fe₃O₇@SiO₂ NPs can be prevented by surface modification of the silica-coated product with a silane coupling agent containing an imidazolium cationic moiety prior to wash steps. In our study, Fe₃O₇@SiO₂ NP products were collected and washed simply using EtOH. This, however, could be facilitating the sol-gel reaction between particles, contributing to additional aggregation. Further investigations with PEG-silane and/or amine- or thiol-based silane coupling agents that can be added directly to the microemulsion synthesis before wash steps may help alleviate Fe₃O₇@SiO₂ NP aggregation. Moreover, the Narita et al. study also suggests that surface-modification post-synthesis may not be effective because the silica-coated NPs are already too aggregated after wash steps.

Finally, additional characterization methods should be investigated. Although DLS data on bare SPIOs are limited by inter-particle aggregation, DLS has been successfully performed on monodisperse suspensions of PEGylated and silica-coated SPIOs to obtain hydrodynamic size information. The first step to improve DLS measurements will be to limit aggregation in the silica synthesis. DLS may also serve as a useful tool to monitor aggregation of NPs by measuring a quantity called the polydispersity index (PDI). Moreover, TEM analysis is not particularly relevant for assessing solution-based chemistry, which is another reason to pursue and optimize DLS measurements. Another popular characterization method of SPIOs in literature is to obtain magnetic hysteresis curves of samples like those shown in Figure 11. Measurements of magnetic susceptibility can be obtained in this way using a SQUID (superconducting quantum interference device) magnetometer. Changes in B vs. M curves can then be correlated with
changes in size and/or surface chemistry of NPs. Moreover, these data can be used in conjunction with relaxivity data since the magnetic moment $\mu$ can be back-calculated from the $B$ vs. $M$ curve\textsuperscript{48} and $R_2 \propto \mu^2$ according to equation (9).

The project offers many avenues to continue exploring an improving. Once small, monodisperse, and de-aggregated Fe$_x$O$_y$@SiO$_2$ NPs are optimally synthesized, either a thin layer of gold can be deposited onto the silica\textsuperscript{7} to take advantage of previously well-studied ligand-gold interactions in the Flynn lab\textsuperscript{29} or functional moieties can be directly attached to the hydroxyl-rich silica surface by, for example, introducing a biotinylated-PEG species to serve as an Avidin-biotin “glue” anchoring site for anti-tumor and targeting groups.
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