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Magnetic nanomaterials for applications in magnetic resonance imaging and cancer stem cell biology

by

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APPROVED, THESIS COMMITTEE

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ABSTRACT

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Magnetic nanomaterials are uniquely suited for applications in biology and medicine. With size compatibility, tunable physical properties, and the capacity for external magnetic control, nanoscale magnetic particles have been exploited for drug delivery, hyperthermia-mediated cancer therapy, cell separation and isolation, and magnetic resonance imaging (MRI). This work explores several different nano-based materials for MRI and cancer cell isolation applications. First, Gd$^{3+}$-loaded carbon nanotube capsules, or gadonanotubes (GNTs), have been analyzed by X-ray absorption spectroscopy to account for the structural contributions to their high MRI contrast enhancement properties. This work revealed the existence of small [Gd-O$_9$] sites in the GNTs with short Gd-O (and thus Gd-H) bond lengths, which contribute to their high performance. Secondly, two new nanomaterials were developed, by loading paramagnetic Mn$^{2+}$ ions into or onto ultra-short single-walled carbon nanotubes (US-tubes) or GNTs (manganonanotubes and manganogadonanotubes, MNTs and MGTs, respectively). With relaxivity ($r_1$) values of 65 (MNT), 74 (MGT), and 110 (GNT) mM$^{-1}$s$^{-1}$ per ion and approximately 13-fold contrast enhancements in all cases over the free ions, US-tubes have been further confirmed as a universal platform for the enhancement of MRI contrast agent properties with the GNTs being the best $r_1$ agents developed to date. Finally, a
method for the isolation of quiescent breast cancer cells has been developed, using iron oxide nanoparticles (IONs) as intracellular labels. Once isolated, functional assays were employed to characterize the drug resistance and stem-like nature of the quiescent subpopulation. The project has thus demonstrated how a magnetic nanomaterial-facilitated procedure can be exploited to probe fundamental questions in cancer stem cell biology.
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<td>CA(s)</td>
<td>contrast agent(s)</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein diacetate, succinimidyl ester</td>
</tr>
<tr>
<td>CN</td>
<td>coordination number</td>
</tr>
<tr>
<td>CSC(s)</td>
<td>cancer stem cell(s)</td>
</tr>
<tr>
<td>CTV</td>
<td>cell tracking velocimetry</td>
</tr>
<tr>
<td>DAPI</td>
<td>diamidino phenylindole</td>
</tr>
<tr>
<td>DI</td>
<td>deionized</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
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<td>DTPA</td>
<td>diethylenetriamine pentacetate</td>
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<td>DPDP</td>
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<td>GNT(s)</td>
<td>gadonanotube(s)</td>
</tr>
<tr>
<td>G1/G2</td>
<td>Gap 1/Gap 2</td>
</tr>
<tr>
<td>HP-DO3A</td>
<td>hydroxypropyl tetraazacyclododecane triacetic acid</td>
</tr>
<tr>
<td>HRTEM</td>
<td>high-resolution transmission electron microscopy</td>
</tr>
<tr>
<td>ICP</td>
<td>inductively-coupled plasma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>ION(s)</td>
<td>iron-oxide nanoparticle(s)</td>
</tr>
<tr>
<td>MACS</td>
<td>magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MEGM</td>
<td>mammosphere epithelial growth medium</td>
</tr>
<tr>
<td>MGT(s)</td>
<td>manganogadonanotube(s)</td>
</tr>
<tr>
<td>MNT(s)</td>
<td>manganonanotube(s)</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MSFE</td>
<td>mammosphere formation efficiency</td>
</tr>
<tr>
<td>ND</td>
<td>nanodiamond</td>
</tr>
<tr>
<td>NMRD</td>
<td>nuclear magnetic resonance dispersion</td>
</tr>
<tr>
<td>NMV</td>
<td>net magnetization vector</td>
</tr>
<tr>
<td>NSF</td>
<td>nephrogenic systemic fibrosis</td>
</tr>
<tr>
<td>OES</td>
<td>optical emission spectroscopy</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>Q</td>
<td>hydration number</td>
</tr>
<tr>
<td>R</td>
<td>bond distance</td>
</tr>
<tr>
<td>$r_1/r_2$</td>
<td>relaxivity</td>
</tr>
<tr>
<td>RF</td>
<td>radio-frequency</td>
</tr>
<tr>
<td>S</td>
<td>synthesis</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SWNT(s)</td>
<td>single-walled carbon nanotube(s)</td>
</tr>
<tr>
<td>$T_1/T_2$</td>
<td>longitudinal/transverse recovery time</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TI</td>
<td>inversion time</td>
</tr>
<tr>
<td>TIC(s)</td>
<td>tumor-initiating cell(s)</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>US-tube(s)</td>
<td>ultra-short single-walled carbon nanotube(s)</td>
</tr>
<tr>
<td>XANES</td>
<td>X-ray absorption near edge structure</td>
</tr>
<tr>
<td>XAS</td>
<td>X-ray absorption spectroscopy</td>
</tr>
<tr>
<td>$\tau_m$</td>
<td>mean residence lifetime</td>
</tr>
<tr>
<td>$\tau_R$</td>
<td>rotational correlation time</td>
</tr>
<tr>
<td>$\chi$</td>
<td>magnetic susceptibility</td>
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</table>
Chapter 1

Introduction

1.1. Magnetic nanoparticles in biology

The prefix “nano,” meaning $10^{-9}$, refers not only to a length scale of one billionth to ten millionth of a meter (1-100 nm), but implies unique properties arising from confinement of matter to these dimensions.¹ Nanoparticles often exhibit physical properties, such as light absorption and emission, reactivity, and magnetism, vastly different from their bulk counterparts. In nanotechnology’s rise to prominence in the years following Richard Feynman’s famous lecture “There’s Plenty of Room at the Bottom,”² researchers have developed nanomaterials of countless different compositions and shapes and rigorously explored their physical, chemical, and biological properties. With many syntheses now well-established, exploiting the unique character of these tiny particles has become a major area of exploration and achievement.
The field of biology, and medicine in particular, is a prime target for nano-based applications. Biological structures, including viruses (20-450 nm), proteins (5-50 nm), and genes (2 nm wide, 10-100 nm long), naturally occur on the nanoscale, offering a perfect interface for engineered structures of similar dimensions.\(^3\) This compatibility has proven useful in many ways, from cellular tagging and imaging with fluorescent semiconducting nanoparticles (quantum dots)\(^4\) to potential therapies such as nanoparticle-facilitated hyperthermia for the destruction of cancerous tissues.\(^5\) A major attraction of nanomaterials is their conduciveness to functionalization. By attaching carefully selected moieties, nanoparticles have the possibility of being directed to specific biological targets.\(^6\) When the nanoparticles are composed of magnetic materials, they have the added benefit of controllable movement through an external magnetic field gradient, and thus have been extensively developed for biomedical applications.\(^3\)\(-\)\(^9\)

Materials may be classified as diamagnetic, paramagnetic, ferro-/ferrimagnetic, or superparamagnetic, based on their magnetic susceptibility ($\chi$), which is the ratio of magnetization ($M$) in response to an applied magnetic field ($H$). For diamagnetic materials, $\chi$ ranges from $-10^{-6}$ to $-10^{-3}$, the negative value indicating that they are repelled from the applied field. For the purposes of this work, diamagnetic materials are not considered to be “magnetic,” which here implies a substantial positive response to an applied magnetic field. Materials with unpaired electrons are paramagnetic and possess a small, positive susceptibility ranging from $10^{-6}$ to $10^{-1}$, being slightly attracted to the field. Ferro- and ferrimagnetic materials have magnetic domains due to coupling between electrons within the material, resulting in positive $\chi$ values. In ferromagnets, the magnetic dipoles within each domain are aligned parallel to one another. In antiferromagnets, the
magnetic dipoles are oriented in opposite directions; and in ferrimagnets, the dipoles are also aligned in opposite directions, and have different magnitudes. The $M$ versus $H$ plot for ferromagnets exhibits a characteristic hysteresis loop, and the $\chi$ values are generally two to three orders of magnitude higher than those of paramagnetic materials. The size of the ferromagnetic particle affects the shape of its hysteresis loop: micron-sized particles or larger have narrow loops, and smaller particles have broad loops. In even smaller ferri- or ferromagnetic nanoparticles, the material is restricted to a single magnetic domain, giving rise to superparamagnetism. Superparamagnetic particles have large magnetic moments and susceptibilities, but do not retain magnetism in the absence of an applied field. They have been extensively explored for biological applications.\textsuperscript{3,9}

Many different types of magnetic nanoparticles have been developed. These include superparamagnetic iron oxide nanoparticles, superparamagnetic or ferromagnetic iron, nickel, and cobalt metallic or core-shell nanoparticles,\textsuperscript{10–13} and antiferromagnetic paramagnetic manganese oxide\textsuperscript{14} and gadolinium oxide nanoparticles,\textsuperscript{15,16} and carbon nanotubes, which often have magnetic properties due to remnant metal catalyst particles from their synthetic processing.\textsuperscript{17} The biological compatibility of these materials depends on many factors, including their size, shape, composition, and coating. Organosilane-coated iron oxide nanoparticles, for example, are nontoxic and can be metabolized by the body and incorporated into red blood cells.\textsuperscript{18,19} Uncoated metallic nanoparticles, on the other hand, are chemically unstable, and therefore less conducive to biological studies. In terms of size, particles larger than 200 nm tend to accumulate in the spleen, while those smaller than 5.5 nm tend to be cleared through the kidneys.\textsuperscript{9,20,21}
Magnetic nanoparticles have been implemented experimentally in several different fields, including drug delivery, magnetically-induced hyperthermia for cancer therapy, magnetic resonance imaging (MRI) contrast and nuclear magnetic resonance-based biosensing, magnetic twisting cytometry, tissue engineering, and magnetic cell separation. Of these applications, two that have become commercially established are magnetic separation and MRI. These areas have been the focus for the magnetic nanoparticles described in this dissertation.

1.1.1. Magnetic nanoparticles in MRI

Magnetic resonance imaging (MRI) relies on the bulk magnetic properties arising from the combined nuclear spins on quintillions of water protons found in biological tissue. In the presence of an applied magnetic field $B_0$, these spins align, creating a net magnetization vector (NMV) parallel to $B_0$, assigned as the $z$ direction. When a 90° radio-frequency (RF) pulse is applied, the NMV is tipped into the $xy$ plane perpendicular to the applied magnetic field. When the pulse is removed, it relaxes back to its original position, by recovery in the longitudinal ($z$) direction and decay in the transverse ($xy$) direction. Coiled wires in the MRI scanner detect and amplify the signal produced by the NMV as it relaxes. The rate of this relaxation is characterized by times $T_1$ for longitudinal recovery and $T_2$ for transverse decay, and these rates are sensitive to surrounding tissue environment. Differences in the environment, such as proton density, give rise to differences in $T_1$ and $T_2$, which in turn are exploited to give contrast in the image. In the presence of a paramagnetic or superparamagnetic material, the relaxation times are shortened substantially, thereby enhancing the contrast in the region of the magnetic material. These materials can thus be employed as MRI contrast agents (CAs). If an MRI
CA predominantly affects $T_1$, it produces a brightening of the image; if it predominantly affects $T_2$, it produces a darkening of the image.

The most widely studied magnetic nanomaterial as an MRI contrast agent is the iron oxide nanoparticle, which has even been commercialized in its dextran-coated form as the FDA-approved Feridex I.V.® (Advanced Magnetics). This is a $T_2$-weighted MRI CA indicated for the detection of liver lesions. Most clinical $T_1$ agents are comprised of paramagnetic ions bound by chelates to protect the body from their toxicity. Gd$^{3+}$, with 7 unpaired electrons and a symmetrical electronic ground state, is the best element for this purpose, despite its toxicity as a free ion. Such paramagnetic substances have the advantage of producing bright contrast in $T_1$-weighted images. For this reason, researchers have been developing nanomaterials incorporating Gd$^{3+}$ and other paramagnetic ions for diagnostic imaging. These include gadolinium oxide nanoparticles, manganese oxide nanoparticles, dysprosium oxide nanoparticles (though $T_2$ effects dominate), and Gd$^{3+}$-loaded ultra-short single-walled carbon nanotubes, or gadonanotubes (GNTs). GNTs have extraordinary contrast enhancement abilities, the sources of which are discussed in this work and others.

1.1.2. Magnetic cell separation

Magnetic cell separation, used to isolate (or deplete) a cell population of interest from a mixture of cells, generally involves two main parts. First, magnetic microparticles or nanoparticles, usually iron oxide particles coated with a biocompatible shell, are targeted to the chosen cell type through specific antibody-antigen interactions. Then, magnetically-labeled cells are separated out from the mixture with some kind of
externally-applied magnetic field. A variety of magnetic separators designs have been developed for this purpose, including the application of a permanent magnetic on the wall of the container, which is the basis for Invitrogen’s DynaBeads®, STEMCELL Technologies’ EasySep™ and R&D System’s MagCellect™, a column packed with magnetizable wires or beads, commercialized by Miltenyi Biotec as MACS® Microbead Technology, and a quadrupole field-flow fractionation device. Magnetic cell separation can theoretically be used to isolate any cell type for which effective immunolabels have been established, such as B cells from whole blood, dendritic cells from mouse tissue, NK cells from whole blood, but are generally more useful in well-defined mixtures. In the work described in Chapter 4, a MACS® column apparatus has been adapted for the isolation of quiescent breast cancer cells internally labeled with dextran-coated iron oxide nanoparticles (IONs).

1.2. Organization

This dissertation consists of two major project areas, one exploring carbon nanotube-based materials as potential magnetic resonance imaging (MRI) contrast agents (Chapters 2 and 3), and the second developing applications in fundamental breast cancer biology (Chapters 4 and 5). In Chapter 2, the structural features contributing to high performance of the GNTs have been probed by X-ray absorption spectroscopy. Chapter 3 describes two new nanomaterials developed by loading paramagnetic Mn²⁺ ions into or onto ultra-short single-walled carbon nanotubes or GNTs (manganonantubes and manganogadonanotubes, MNTs and MGTs, respectively). MNTs and MGTs were evaluated in terms of their MRI contrast enhancement abilities. In Chapter 4, GNTs and
IONs were tested as agents for the magnetic isolation of quiescent breast cancer cells, and Chapter 5 illustrates how this method was exploited to probe the fundamental biology of quiescent cell populations. These endeavors are united by the theme of nanoscale science and technology in medicine.
Chapter 2

The structure-performance relationship in gadonanotubes

2.1. Introduction*

Magnetic resonance imaging (MRI) is a powerful, minimally invasive clinical technique to evaluate the inner workings of the human body. Relying on the inherent nuclear spin of protons found throughout patient tissue, MRI resolves details including anatomical features and blood flow. Within the past two decades, the use of diagnostic

* Portions of this chapter are adapted or reproduced from:

MRI scans has substantially increased, with an annual growth of 10% from 1996 to 2010 in six large integrated healthcare systems analyzed in one study. While MRI performance continues to improve with new technologies and higher magnetic field strengths, the use of contrast agents (CAs) to enhance imaging quality remains prevalent, supplementing 25-30% of all MRI scans. In this chapter, the focus lies exclusively on paramagnetic Gd$^{3+}$-based MRI CAs and the fundamental properties that affect their performance.

As introduced in Chapter 1, if a paramagnetic material, such as the Gd$^{3+}$ ion, is present in an MRI sample, the unpaired electron spins interact with the nuclear spins of the protons, thus shortening the rate of time for the NMV to relax back to its original position. Differences between $T_1$ relaxation rates in the vicinity of the CA create a contrast with surrounding tissue that can be translated into a brighter $T_1$ image in the region of interest.

Longitudinal relaxivity ($r_1$) describes the efficacy with which a paramagnetic entity enhances the relaxation of neighboring water protons once the RF pulse has been removed. Relaxivities are calculated as the difference in inverse relaxation times between the solvent environment (namely water) and the contrast agent, normalized by the concentration of the agent [CA] (Equation 2.1).
Important factors influencing relaxivity are the electronic properties of the paramagnetic substance, the number of water molecules directly coordinated to the ion, called hydration number \((Q)\), the rotational correlation time of the water-CA complex \((\tau_R)\), the CA-proton distance \((R_{\text{Gd-H}})\), and the mean residence lifetime of coordinated waters \((\tau_m)\), which can be directly coordinated (inner sphere), hydrogen-bonded to ligands on the contrast agent (second-coordination sphere), or in the bulk solvent (outer sphere).

With 7 unpaired electrons (the highest number for any ion), Gd\(^{3+}\) has a large magnetic moment \((\mu^2 = 63 \ \mu_B^2)\), a symmetric electronic ground state, and rapid exchange between coordinated and bulk water molecules. These properties render it the most prevalent paramagnetic ion in clinical MRI CAs. Five of seven currently FDA-approved MRI CAs are Gd-based.\(^{35}\) However, free Gd\(^{3+}\) is toxic and insoluble at biological pH, and thus must be sequestered from direct contact with human tissue. In clinical MRI CAs, most Gd\(^{3+}\) ions are chelated, as in gadolinium diethyleneetriamine pentacetate (Gd-DTPA, Magnevist®) and gadolinium 10-(2-hydroxypropyl)-1,4,7-tetraazacyclododecane-1,4,7-triacetic acid\(^36\) (Gd-HP-DO3A, Prohance®).

\[
 r_1[\text{CA}] = \left( \frac{1}{T_1} \right)_{\text{CA}} - \left( \frac{1}{T_1} \right)_{\text{solv}}
\]

Equation 2.1 Experimental determination of relaxivity
The discovery of gadonanotubes (GNTs) in 2005 brought to light a new, highly effective method for sequestering $\text{Gd}^{3+}$ ions: loading them ultra-short single-walled carbon nanotube (US-tube) capsules. Single-walled carbon nanotubes (SWNTs) are long (on the order of microns), high aspect-ratio (diameter of a few nanometers) tubes with a wall composed of aromatic carbon rings, as in graphene. US-tubes are produced through fluorination and pyrolysis of SWNTs, yielding much shorter (20-80 nm) carbon capsules. In GNTs, not only were the $\text{Gd}^{3+}$ ions tightly bound to the US-tubes, preventing any leakage, but the relaxivities were the highest reported per $\text{Gd}^{3+}$ ion: 170 mM$^{-1}$s$^{-1}$. Magnevist®, for comparison, has $r_1 \sim 3.3$ mM$^{-1}$s$^{-1}$, about 50 times lower. While others have extensively reviewed the various kinds and types of paramagnetic MRI contrast agents, this chapter explores the factors contributing to the exceptional performance of the GNTs.

### 2.2. Background: Relaxivity studies of GNTs

Table 2.1 lists the $r_1$ values of GNTs compared to other relevant $\text{Gd}^{3+}$-based MRI CAs (Gd-CAs). A more detailed analysis of the reported results begins to shed light on the superiority of GNTs over other agents.
Table 2.1 Gd$^{3+}$-based MRI CAs and their relaxivities at 25 °C*, 1.41-1.5 T

<table>
<thead>
<tr>
<th>Contrast Agent</th>
<th>Relaxivity ($r_1$, s$^{-1}$mM$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GdCl$_3$</td>
<td>2.9 ± 0.8</td>
<td>Law 2014</td>
</tr>
<tr>
<td>Gd(thd)$_3$@US-tubes</td>
<td>26.6 ± 6.6 (2.2 ± 2.5 free chelate)</td>
<td>Law 2014**</td>
</tr>
<tr>
<td>Gd(acac)$_3$@US-tubes</td>
<td>103.0 ±16.1 (1.1 ± 0.7 free chelate)</td>
<td>Law 2014**</td>
</tr>
<tr>
<td>Gd(hfac)$_3$@US-tubes</td>
<td>105.9 ± 1.5 (0.9 ± 0.3 free chelate)</td>
<td>Law 2014**</td>
</tr>
<tr>
<td>Gd-ND</td>
<td>58.82 ±1.18 (37 °C) (5.42 ± 0.20 free)</td>
<td>Meade 2010</td>
</tr>
<tr>
<td>Gd-DTPA (Magnevist®)</td>
<td>3.3 ± 0.2</td>
<td>Rohrer 2005$^{41}$</td>
</tr>
<tr>
<td>GNT</td>
<td>170 (40 °C)</td>
<td>Sitharaman 2005</td>
</tr>
</tbody>
</table>

* Unless otherwise specified
** Exact values provided by Justin J. Law

2.2.1. US-tube contributions to relaxivity

2.2.1.1. Free radicals

Studies of the nuclear magnetic resonance dispersion (NMRD) profiles of empty US-tubes revealed a surprising result: even empty tubes have substantially shortened $T_1$ values at low frequencies (< 1 MHz), accounting significantly, if not entirely, for the high performance at low fields.$^{42}$ This effect was attributed to high concentrations of free radicals present on the US-tube surface, as characterized by electron spin resonance spectroscopy. Such radicals are likely created by the disruption of the aromatic carbon cage during synthesis of the US-tubes. While a lack of concentration controls made
quantifying the exact Gd\(^{3+}\) contributions impossible, a comparison of the shapes of the curves for GNTs versus US-tubes suggests that US-tubes alone are high performers. Until the concentrations of these radicals are measured and accounted for, the dispersion profile cannot be represented in terms of Gd\(^{3+}\) concentrations. However, at clinical field strengths of 1.5 T, Gd\(^{3+}\) appears to dominate, thus permitting \(r_1\) calculations at this field to be based on Gd\(^{3+}\) alone.

2.2.1.2. Rotational correlation time (\(\tau_R\))

Other carbon nanomaterials have also been tested in Gd-CAs. Development of Gd-nanodiamonds (Gd-ND), for example, isolated the contribution of tumbling rate to the relaxivity of a Gd\(^{3+}\)-chelate complex\(^{43}\). With a chelated Gd\(^{3+}\) ion offering the only source of paramagnetism, comparisons of the chelate to the chelate-ND complex revealed a tenfold \(r_1\) enhancement. Authors attributed this increase to the slower tumbling rate afforded the complex by the bulky ND. GNTs certainly have this benefit as well, although it is not an isolated effect, as other factors contribute. The US-tube cage retards the overall tumbling rate, thus enhancing \(r_1\), though by an unknown degree.

However, in a study measuring the relaxivities of various Gd\(^{3+}\)-chelates loaded into US-tubes, 2,2,6,6-tetramethyl-3,5-heptanedione (thd)-chelated Gd\(^{3+}\) ions, with a likely \(Q\) value of 0, were found to have an \(r_1\) value enhanced by ten times that of the free Gd(thd)\(_3\).\(^{28}\) With no inner sphere contribution, this enhancement may represent a close estimate of the tumbling rate contribution to GNTs and agrees well with results with the NDs. While individual US-tubes could provide reduced tumbling times, large bundles of
hydrophobic US-tubes in suspension are more likely to account for the effect observed in GNTs.

**2.2.2. Hydration number (Q)**

As illustrated in Equation 2.2, the hydration number (Q) in an MRI contrast agent has a direct correlation to the efficacy of the CA.

\[ r_1 = \frac{Q}{55.5} \left( \frac{T_{1p}}{\tau_m} \right)^{-1} \]

Equation 2.2 Relationship between hydration number Q and relaxivity \( r_1 \)

\( T_{1p} \) is the relaxation time of water protons coordinated to the Gd\(^{3+} \) ion. In commercial CAs such as Gd-DTPA and Gd-HP-DO3A the chelate occupies all Gd coordination sites except one, providing a \( Q = 1 \). In GNTs, however, the exact hydration number is unknown. One study to estimate \( Q \) was based on theoretical modeling of the nuclear magnetic resonance dispersion (NMRD) profiles of GadoDex, GNTs solubilized by a dextran coating. Based on these estimations, GNT profiles at higher frequencies (>10 MHz) overlapped with fitting curves modeling a \( Q \) of 2.44.

The Gd\(^{3+}\)-chelate study discussed previously also indirectly explored the \( Q \) value of GNTs. Gd(acac)\(_3\) and Gd(hfac)\(_3\), chelates with an inner sphere water coordination of 2 were compared to Gd(thd)\(_3\), with \( Q = 0 \) (acac = acetylacetone and hfac = hexafluoroacetylacetone). Relaxivities were compared both in the free chelates alone and the chelates loaded into US-tubes (values shown in Table 2.1). The free chelates exhibited such low relaxivities, no significance difference was observed between different chelates. In US-tubes, however, the increased \( Q \) values are apparent, with \( r_1 = \)
26 s⁻¹mM⁻¹ for \( Q = 0 \) and \( r₁ \sim 104 \) s⁻¹mM⁻¹ for \( Q = 2 \). The GNT \( r₁ \) values reported in this study up to 66\% higher than Gd(acac),@US-tubes, implying potentially higher \( Q \) values than previously predicted. Thus, the structural source of the high GNT performance should be explored in greater detail.

2.3. X-ray absorption spectroscopic analysis of GNTs

Despite intensive research into the unique nanoscale environment provided Gd³⁺ ions by the US-tubes, their chemical environment, specifically coordination numbers and exact hydration numbers, have remained unclear. While Gd³⁺ has a potential coordination number of 9, which, in GNTs, could be occupied by water molecules, hydroxides, carboxylates, chloride anions, carbon from the US-tube cage, or neighboring Gd³⁺ ions. In order to probe this chemical environment, X-ray absorption spectroscopy (XAS) was employed.

In XAS, a specimen is irradiated by monochromatic X-ray source, and the X-ray photon energy is continuously changed across an absorption edge of the element of interest. Therefore, a powerful X-ray source provided by a synchrotron storage ring, such as the one at the Advanced Photon Source located at Argonne National Laboratory (Illinois, USA) is needed to carry out the measurements. Synchrotron radiation provides intense polychromatic X-ray photons, whose energy can be selected by a monochromator. When the X-ray energy (\( E \)) is equal to or slightly higher (< 30 eV) than the binding energy (\( E₀ \)) of the core electrons, X-ray absorption events occur and the electrons are excited to the empty bound states. The X-ray absorption measurements in this energy range (near the absorption edge) reveal the electronic structure of the matter.
The information on the charge state of and the local symmetry around the absorbing atom can be obtained. The data recorded in this energy range is called X-ray absorption near edge structure (XANES).

As the X-ray photon energy further increases, the excited electrons will gain enough kinetic energy ($E - E_0$) to leave the absorbing atom and travel as photoelectrons in the matter. These photoelectrons will be scattered by neighboring atoms, and a portion of them will be scattered back to the atom from which they are originated. The forward and backscattered photoelectrons will interfere, thus modulating the absorption cross section in an oscillatory fashion. The frequencies and amplitudes of these oscillations carry the information on the interatomic distances, their spreads, and the coordination numbers around the absorbing atom. Therefore, X-ray absorption measurements extending about 1000 eV above the binding energy reveal the atomic structure around the absorbing atom. This portion of the data is called extended X-ray absorption fine structure (EXAFS).

Depending on sample conditions, X-ray absorption measurements can be carried out through the absorption channel (transmission mode) or the emission (fluorescence mode) channel. The latter originates from the same absorption event. X-ray absorption creates the core holes (such as 2p) in the atom, and filling of these core holes by higher energy electrons (must be the electrons on the d orbital, if the holes have p character) will emit X-ray fluorescence. The probability of fluorescence emission is tied to that of absorption. Therefore, the recorded fluorescence intensities as a function of the X-ray photon energy contain all the aforementioned structural information, either electronic or atomic. XAS is inherently a local structure technique, as the photoelectrons do not travel very far.
Thus, XAS provides valuable structural information as to the target element’s oxidation state and local symmetry (XANES) and coordination number (EXAFS). Since the inner structure of GNTs cannot be probed by X-ray crystallography, XAS is crucial for determining the local environment of Gd\(^{3+}\) ions within US-tubes.

2.4. Methods

2.4.1. XAS measurements

For a heavy element like Gd, the L\(_{1,2,3}\) absorption edges (the transitions of 2s or 2p core electrons to vacuum) range from 7.1 keV to 8.4 KeV and can be readily accessed. X-ray absorption measurements around the Gd L\(_1\) and L\(_3\) edge were carried out at the 5BM-D beamline of DND-CAT, at the Advanced Photon Source (Argonne, IL). Details were provided previously\(^{45}\). The absorption data of dry and suspended GNTs were collected in fluorescence mode. Due to the low concentration of the GNT solution, only the XANES was measured, while the EXAFS data were measured for the dry-GNTs. A measurement at 20 K was also carried out on the dry-GNT sample. Commercial Gd[C\(_2\)H\(_3\)O\(_2\)]\(_3\)•4H\(_2\)O (or GdAc), Gd(OH)\(_3\), and GdCl\(_3\)•6H\(_2\)O (Alfa Aesar) were used as reference compounds. Fine powders of these compounds were spread on strips of Scotch tape®, which were then folded several times to produce sufficient absorption and measured in transmission mode. A 10 mM GdCl\(_3\) solution was placed in a Plexiglas container and measured in fluorescence mode.
2.4.2. GNT synthesis

GNTs were prepared according to previously established procedures. Briefly, full-length single-walled carbon nanotubes produced via the arc discharge method (Carbon Solutions) were fluorinated and pyrolyzed to US-tubes. The US-tubes were purified with 12 M HCl to remove catalytic impurities, sonicated with Na metal in THF to debundle the tubes, and refluxed in 6 M HNO$_3$ for 5 min, with deionized (DI) water washing over a glass frit between each step. Approximately 1 mg/mL of US-tubes were bath sonicated in a 6.7 mM solution of GdCl$_3$·6H$_2$O (Aldrich) for 1 h and then washed to remove unbound Gd$^{3+}$ ions. GNTs were oven-dried and suspended in 0.17% (w/v) Pluronic® F 108NF Prill Poloxamer 338 (BASF) at 1 mg/mL. The sample was spun down at 3200 rpm, collecting the supernatant to remove unsuspended GNTs. Suspended GNTs were dialyzed in DI water to obtain a stable, dilute suspension of 31.2 mM Gd.

2.4.3. X-ray spectroscopic analysis

The XANES is sensitive to the local electronic structure around the Gd$^{3+}$ ion. According to the selection rules for electric dipole radiation, the Gd $L_{2,3}$ edges probe the empty states of the $s$ and $d$ symmetries by the excited $2p_{1/2}$ and $2p_{3/2}$ electrons, respectively, while the Gd $L_1$ edge probes the empty states of the $p$ character by the excited $2s$ electrons. The excited $2s$ electrons may probe as well part of the empty $d$ states that mixes with the ligand $2p$ states under the right conditions, i.e., available empty $d$ states and suitable local geometries. XANES of the GNTs and reference materials is shown in Figure 2.1.
To better understand the XANES data, the simulations were carried out using the FDMNES code based primarily on the local structures of hexagonal Gd(OH)$_3$ (space group: $P6_3/m$, $a = b = 6.329$ Å and $c = 3.631$ Å, $\alpha = \beta = 60^\circ$ and $\gamma = 120^\circ$). Gd(OH)$_3$ allows access to hydrogen bonded to the coordinating oxygen ions. The FDMNES code was developed based on dipolar approximation, both with the full-multiple-scattering (FMS) approach and with the finite difference method (FDM) approach, respectively. The FMS utilizes the spherical muffin-tin (MT) approximation to atomic potentials, whereas the FDM, a non-muffin-tin (NMT) approach, sets atomic potentials on an equally spaced 3D-grid with $V=0.25\times0.25\times0.25$ Å$^3$. The FDM approach is thus sensitive to variations in atomic potentials. The FMS approach, however, is rapid and produces results largely similar to those obtained by the FDM approach in terms of consistency. The results presented here are obtained with the FMS method.

The EXAFS probes the local atomic arrangement around the Gd$^{3+}$ ion. The data were processed and analyzed using the Athena package. As is well known, the EXAFS is described by $\chi(k) = S_0^2 \sum_i N_i |f_i(k)| \sin [2R_i k + \varphi_i(k)] + \frac{4}{3} C_3 k^3 e^{-2\sigma^2 k^2}$. Both the amplitudes $|f(k)|$ and phases (the $\sin$ term) are sensitive to the details of the local structure about the absorbing atoms, i.e., Gd. $\varphi(k)$ is the phase shift due to scattering of the photoelectrons, and the $C_3$-containing term is a correction to the phase due to the off-harmonic characteristic in the bond distance fluctuation. The Fourier transform (FT) of $\chi(k)$ produce the atomic pair interaction at various lengths. Figure 2.4 shows the $\chi(k)$ data (right) of the GNT and GdCl$_3$ as well as the FTs (left) of $\chi(k)$. To determine the coordination number (CN) and bond distance $R$, the phase and amplitude were generated by the FEFF code based on a reference material. Using these phases and amplitudes to fit
the reference EXAFS data produces a scaling factor, $S_0^2$, called the amplitude reduction factor that is predominantly related to the absorbing atom.\textsuperscript{49} As determined from the fittings of the EXAFS data of the reference materials, $S_0^2$ is essentially equal to 1, and is assumed to be transferable to the GNT systems in this study. The Debye-Waller factor $e^{-2\sigma_i^2 k^2}$, which describes the bond disorder, was also determined, where $\sigma_i^2$ is the root mean square of the bond distance fluctuation about the bond distance $R$.

2.5. Results & Discussion

2.5.1. Small Gd sites and atomic arrangement in GNTs

In order to estimate the size of clusters of Gd$^{3+}$ ions within the US-tubes, theoretical XANES spectra were calculated for clusters based on Gd(OH)$_3$ of increasing size (Figure 2.2). As the clusters become larger, a small peak appears about 10 eV above the “whiteline,” the term for the intense absorption observed in the near-edge region of the spectrum. As this peak is absent from the XANES spectrum of the GNTs (Figure 2.1), any Gd$^{3+}$ clusters present are presumed to be small. The constraints of the US-tube cage (14 Å in diameter) could explain this observed size restriction.

A closer look at the effect of cluster size on the XANES traces reveals a slight increase in peak asymmetry upon each additional attachment of hydrogen atoms (Figure 2.2b). The bonding environment around the Gd$^{3+}$ ion becomes more delocalized, which can also explain the asymmetry of the suspended GNT whiteline compared to dry GNTs (Figure 2.1). As can be seen in the XANES spectra of fresh (31.2 μM GNTs A) versus old (31.2 μM GNTs B) suspensions of GNTs, a marked change in peak position occurs
over time. However, an increase in hydrogen bonding cannot fully explain such a significant change in the absorption edge intensity. Such an effect was shown to be small in Figure 2.2. The peak position change could have been attributed to a rebundling of the GNTs in suspension over time, but bundled versus debundled GNTs did not exhibit the XANES behavior noted for fresh compared to aged GNTs. Model compound GdAc (Gd(C₂H₃O₂)₃•4H₂O) had a similar shift in whiteline intensity compared to GdCl₃•6H₂O due to disordering of Gd-O bonding for GdAc. This suggests that as GNT suspensions age, the Gd-O bonds also undergo an atomic rearrangement or disordering.
Figure 2.1 The XANES spectra of 10-mM GdCl$_3$ (top), GdAc (next from top), dry GNTs (full lines, third from top), fresh 31.2-μM GNTs (A) (solid circle), Gd(OH)$_3$ (full line, bottom) and aged 31.2-M GNTs (B) (open circle).
Figure 2.2 (a) Full-multiple-scattering (FMS) calculations of the Gd $L_z$ edge using a cluster complex generated from Gd(OH)$_3$. The cluster size was increased by adding a shell, and [M] = Gd$_{9}$O$_{9}$H$_{6}$. Cluster size increases from 2.5 to 4.1 Å. The arrow indicates a small peak that arises with a larger cluster size. (b) Detailed comparison of the FMS calculations for GdO$_9$, GdO$_9$H$_6$, and GdO$_9$H$_6$H$_6$ clusters shown in (a). Curves 1 and 2 are the differences of the GdO$_9$H$_6$ and GdO$_9$H$_6$H$_6$ results, respectively, to the GdO$_9$ result. They are presented to show the changes in the peak symmetry.
2.5.2. Short Gd-H distances

Fitting of the EXAFS data of the first neighbor coordination in dry GNTs revealed the likelihood of bimodal Gd-O bond distances (Figure 2.3). Fittings of the data with single Gd-O bond distances (traces a and b) had poorer correlations than the fits with two discrete Gd-O distances (trace c). Even more detailed fitting results (Table 2.2) estimated the Gd-O bond distances as 2.24 Å and 2.41 Å. These bond distances are shorter than the 2.463 Å Gd-O distances found in clinical Gd CAs or the 2.44 Å found for Gd(OH)₃.

As the oxygen atoms are likely hydrogenated (hydroxide moieties or water molecules), the shorter Gd-O bonds in GNTs indicate shorter Gd-H distances ($R_{\text{Gd-H}}$) than the clinical CAs as well. Narten and Hahn determined in their neutron scattering studies of relatively concentrated NdCl₃ solutions (2.85 M) (in which the Nd³⁺ ions are fully hydrated) that the Nd³⁺ ion is coordinated by 8.5 H₂O with $R_{\text{Nd-O}}$ and $R_{\text{Nd-HD}}$ distances of 2.48 and 3.13 Å. In Cossy et al. the Nd³⁺ ion is coordinated by 9 water molecules in dilute solutions, with $R_{\text{Nd-O}} = 2.51$ and $R_{\text{Nd-H}} = 3.14$ Å, while the Sm³⁺ ion is coordinated by 8.5 waters with $R_{\text{Sm-O}} = 2.46$ and $R_{\text{Sm-H}} = 3.12$ Å. The tilt angles from neutron data averaged around 26 ± 4° for Nd³⁺, Sm³⁺, Dy³⁺ and Yb³⁺. They suggested a crossover of the N from 9 to 8 around Gd. The results obtained here for 10-mM GdCl₃ fit well with this prediction ($R_{\text{Gd-O}} = 2.41$ Å and CN = 8.6) (Table 2.2). Given the similar CN, $R_{\text{Gd-H}} = 3.06$ Å may be deduced from the M-O-H angle (M = Nd or Sm, 124.6±1.3°). Similarly, $R_{\text{Gd-H}}$ is calculated to be 2.90 Å for $R_{\text{Gd-O}} = 2.24$ Å and 3.05 Å for $R_{\text{Gd-O}} = 2.41$ Å, respectively, for the GNTs. Based on similar considerations, $R_{\text{Gd-H}} = 3.11$ Å for $R_{\text{Gd-O}} =$
2.463 Å for the clinical agent.\textsuperscript{50} This difference could affect \( r \) since it is proportional to \( 1/R_{\text{Gd-H}}^6 \). For \( R_{\text{Gd-O}} = 2.24 \) Å, GNTs could have \( r \) greater by 52\%, relative to our measurements of Gd(OH)\(_3\),\textsuperscript{50} and greater by 66\% compared to the Gd-OH\(_2\) distance, where the Gd-O bond distance was 2.50 Å\textsuperscript{53} for which the Gd-H distance was \( \sim 3.16 \) Å. Based on these estimates and the measured Gd-O bond distances, the short \( R_{\text{Gd-H}} \) likely constitutes major contributions to GNT performance.

![EXAFS fits](image)

Figure 2.3 Best fits of the EXAFS of the first neighbor coordination in 10-mM GdCl\(_3\), (Upper) and dry GNTs (Lower). For dry GNTs, three fits are shown: a) one Gd-O distance, b) one Gd-O distance with the \( 4C/k^3 \) term included, and c) two Gd-O distances, respectively. See Table 2.2 for details.
Table 2.2 First coordination sphere around Gd$^{3+}$ ($S_0^2 = 1$)*

<table>
<thead>
<tr>
<th>Samples</th>
<th>R(Å)</th>
<th>$\sigma^2$(Å$^2$)</th>
<th>CN</th>
<th>$C_3 \times 10^{-4}$</th>
<th>R-factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNTs</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>c</td>
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<td>0.0045</td>
<td>2.4</td>
<td>0</td>
<td>0.0011</td>
</tr>
<tr>
<td></td>
<td>2.41</td>
<td></td>
<td>6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
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<td>0.0105</td>
<td>8.6</td>
<td>-0.0013</td>
<td>0.004</td>
</tr>
<tr>
<td>a</td>
<td>2.41</td>
<td>0.0110</td>
<td>8.7</td>
<td>0</td>
<td>0.011</td>
</tr>
<tr>
<td>10-mM GdCl$_3$</td>
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<td>0.0074</td>
<td>8.6</td>
<td>0</td>
<td>0.0033</td>
</tr>
<tr>
<td>Gd(OH)$_3$</td>
<td>2.44</td>
<td>0.0103</td>
<td>8.4</td>
<td>0</td>
<td>0.0026</td>
</tr>
</tbody>
</table>

*Data range of $k = 2.5 - 10$ Å$^{-1}$ and $R = 1 - 2.4$ Å are used.

### 2.5.3. Gd-Gd and Gd-C interactions

X-ray spectroscopy and detailed modeling were employed to explore potential Gd-Gd interactions and Gd-C interactions in GNTs. As shown by the EXAFS data for dry GNTs and GdCl$_3$, interactions beyond the first coordination sphere are weak for both (Figure 2.4). This can be seen by the weak peaks beyond 2.5 Å in the FTs of the data. In GdAc, there is a Gd-Gd interaction near 4 Å, as shown in the FT of the EXAFS data (Figure 2.5). Since dry GNTs exhibit a similar chemical environment to GdAc, as shown in the first derivatives of the Gd $L_1$ edge XANES (Figure 2.6), the FTs of the GdAc and GNT EXAFS were used to estimate the Gd-Gd interactions in GNTs as well. After accounting for multi-scattering, which has a contribution to peaks beyond the first coordination sphere at 2 Å, it was inferred that there are Gd-Gd interactions in dry GNTs at a distance of 4.2 Å.
Figure 2.4 The $k^3$-weighted EXAFS data (Left) and their FTs (Right) of dry GNTs and 10-mM GdCl$_3$, measured in fluorescence mode. Dots are the data from 31.2-µM GNTs suspension. The arrow indicates a multiple electron excitation.
Figure 2.5 Fourier transforms of the EXAFS data for GdAc and GNT. Also shown are the fitting results (dash lines) of the data up to 4.5 Å.
Any interaction between Gd and the carbon cage in the GNTs remains unclear from the data. However, the bimodal Gd-O bond distribution discussed above could be the result of the US-tube exerting an asymmetrical force field on the small Gd clusters. In suspension, the drop in whiteline intensity (Figure 2.1) may be caused by Gd-O bonds being more discretely distributed.

The US-tube has a measured average diameter of 14 Å.\textsuperscript{54} The lack of Gd-C bonding gives further credence to the small size of the [Gd-O\textsubscript{n}] sites in the GNTs that was postulated above due to lack of a peak 10 eV above the whiteline in the XANES traces.

Figure 2.6 First derivatives of the Gd $L_\ell$ edge XANES spectra measured on 10-mM GdCl\textsubscript{3}, dry-GNTs, and GdAc
(Figure 2.1). Since \( R_{c-o} \sim 1.5 \) Å for a single C-O bond, the \([\text{Gd-O}_9]\) site diameter was estimated as \( d \sim 11 \) Å. Due to steric hindrances in the tube, it is unlikely that Gd sites can pass one another. Thus, it is possible that a single water molecule in the US-tube could be simultaneously relaxed by both Gd ions separated by 4.2 Å. Although speculative, this double relaxation may further enhance the GNT relaxivities.

2.5.4. Potentially high \( Q \) values

The lack of any strong Gd-C interaction in the GNTs, the small Gd cluster size, the relatively large US-tube diameter, and the short Gd-O (and therefore Gd-H) distances leave ample room and indication for a large number of coordinating oxygen ions interacting with water protons. Further corroborating this concept, the Gd \( L_3 \) edge shape for suspended 31.2 \( \mu \)M GNTs is best modeled by attaching six protons to the Gd-coordinated oxygen ions. If accurate, this represents a crucial contribution to the remarkably high relaxivity values of GNTs. As shown in Equation 2.1, \( r_1 \) is directly proportional to the number of water molecules coordinated to the Gd\(^{3+}\) ion.

2.6. Conclusions

Gadonanotubes, with \( r_1 \) values as high as 170 mM\(^{-1}\)s\(^{-1}\), are among the highest-performing Gd-based MRI CAs developed to date (on a per-Gd\(^{3+}\) ion basis). As prevailing theory cannot adequately predict their behavior, much research has gone into probing the structural origins of their efficacy. Previous studies have partially accounted for GNT contrast behavior, pointing to the slower tumbling rates afforded by the relatively large carbon cage\(^{27}\) and/or bundles of CNTs in suspension and radicals present
on the CNTs that contribute to high relaxivities at low field strengths. In addition, theory has estimated GadoDex to have a hydration number of 2, but a comparison of Gd-chelates@US-tubes has indicated that \( Q \) could be higher.

Building on this earlier work, X-ray absorption spectroscopy has revealed new information as to the structural origin of the high performance of GNTs as potential MRI CAs. First, \( \text{Gd}^{3+} \) ions were shown to be coordinated by \( \sim 9 \) oxygen ions, leading to potentially high \( Q \) values (up to six), even higher than predicted. Secondly, Small [Gd-O\(_9\)] sites and the lack of detectable Gd-C interactions depict a structural environment conducive for water protons to be relaxed by more than one \( \text{Gd}^{3+} \) ion simultaneously. Finally, measured Gd-O bond distances were discovered to be shorter than for well-studied clinical agents. These diminished dimensions lead to unusually short Gd-H distances for the GNTs. Since \( r_1 \) scales as \( 1/R^6 \), this is undoubtedly be a major contributor to high relaxivity. Such pertinent revelations, combined with previous discoveries, describe a panoply of structural facets leading to this extraordinary MRI contrast agent.
Chapter 3

Manganonanotubes and manganogadonanotubes as MRI contrast agents

3.1. Introduction

The extraordinary performance of gadonanotubes (GNTs) as described in Chapter 2 raises questions about the contributions of the unique US-tube environment, which has motivated researchers to push for higher performance. As discussed, GNTs could have a hydration number ($Q$) larger than two or three. While Gd$^{3+}$ has a maximum coordination number of 9, some of these sites are likely occupied by OH moieties (bridging and nonbridging), which help sequester and anchor the Gd$^{3+}$ ions within the US-tube nanostructure.\textsuperscript{27} One possible mechanism of Gd$^{3+}$ loading is explained by its pH-dependent chemistry. At low pH, GdCl$_3$ exists as free Gd$^{3+}$ ions in solution, completely hydrated by 8-9 water molecules. As the pH is raised toward neutral values, Gd(OH)$_3$
precipitates out. In the presence of US-tubes, Gd$^{3+}$ potentially “polymerizes” into small hydroxy-bridged Gd$^{3+}$ clusters within the US-tubes.$^{27,55}$

High-resolution transmission electron microscopy (HRTEM) imaging has revealed that Gd$^{3+}$ ions are present at intervals along the length of the US-tubes.$^{26}$ This observation has been attributed to the defect sites that exist along the length of the carbon nanotubes as a result of fluorination, pyrolysis, and acid treatment prior to Gd$^{3+}$ loading. Due to the oxidation of the carbon cage with nitric acid, carboxylic acid groups likely exist at these sites$^{56,57}$ and may provide a second mechanism for Gd$^{3+}$ sequestration, through the coordination of Gd$^{3+}$ to the carboxylic acid groups at the defect sites. These two mechanisms of sequestration are not mutually exclusive and may both contribute to the stability of GNTs. Considering the pH chemistry of Gd$^{3+}$, a two-step loading mechanism is also possible. First, the coordination of Gd$^{3+}$ ions to the US-tubes occurs at low pH. Then, as pH is increased through washing with DI water (pH ~5.5), hydroxy-bridges begin to form between the Gd$^{3+}$ ions, irreversibly attaching them to (or within) the US-tubes.

With hydroxy-bridges occupying Gd$^{3+}$ coordination sites, a hydration number of 2, suggested through GadoDex studies,$^{44}$ or 3, suggested through Gd-chelates loaded into US-tubes,$^{28}$ is possible. This fairly low Q value provides an opportunity for improvement in contrast agent design. Since relaxivity is directly proportional to Q (Equation 2.2), raising the hydration number would improve the agent. In order to potentially raise Q, alternative chemistries should be explored. As the first magnetic resonance imaging contrast agent (MRI CA) ever studied$^{58}$ and as a paramagnetic material that has been explored extensively since, the Mn$^{2+}$ ion is the obvious candidate. With 5 unpaired
electrons, the electron spin resonance of Mn$^{2+}$ with the proton spins of coordinated water molecules drastically enhances the relaxation rate in an RF-perturbed sample. The Mn$^{2+}$ ion, however, has very different aqueous chemical properties than the Gd$^{3+}$ ion. Namely, Mn$^{2+}$ remains unhydroxylated at neutral pH, hypothetically leaving more sites available for water coordination. Thus, the goal was to load Mn$^{2+}$ ions into US-tubes and analyze their performance in relation to GNTs as well as other Mn$^{3+}$-based MRI CAs.

In addition to its different chemical nature, the Mn$^{2+}$ ion has several advantages over Gd$^{3+}$ for fundamental and clinical applications. As reviewed by Koretsky and Silva, manganese-based agents can be exploited for their unique biology, including assisting in electron transport, neurotransmitter synthesis, and free radical detoxification. As an MRI CA, Mn$^{2+}$ can exchange with Ca$^{2+}$ and Mg$^{2+}$ and can thus be used to measure processes that involve these metal ions, thereby providing a unique tool for detailed brain mapping.\textsuperscript{60-65} Due to its efficacy, the FDA has approved one Mn$^{2+}$-chelate, manganese dipyridoxyl diphosphate (Mn-DPDP), which is sold under the brand name Teslascan for MR imaging of liver lesions. Liver contrast is reliant on the preferential uptake of Mn$^{2+}$ in the liver upon exchange with Zn$^{2+}$, as Mn$^{2+}$ is released from the chelate.\textsuperscript{66} Such chemistry is not possible or desirable with clinical Gd-based MRI CAs, especially since leakage of Gd$^{3+}$ from chelated forms is highly disadvantageous due to toxic side effects.\textsuperscript{67} In one study comparing liposome-conjugated Mn$^{2+}$ and liposome-conjugated Gd$^{3+}$, Mn$^{2+}$ leaked faster and more substantially than Gd$^{3+}$ ions.\textsuperscript{68} However, it was shown to have higher contrast at lower concentrations in the liver of rats. In another study to image myocardial infarctions in rats, the Mn$^{2+}$-based CAs tested were highly specific and less sensitive to timing (due to slower clearance from the blood pool) than the Gd$^{3+}$-based agents.\textsuperscript{69} In a
comparison between FDA-approved gadolinium diethylenetriaminepentaacetate (Gd-DTPA) versus Mn-DPDP, the Mn\textsuperscript{2+} agent was more effective for the detection of pancreatic lesions.\textsuperscript{70}

Importantly, Mn-DPDP was found to have a significantly better safety factor than Gd-DTPA.\textsuperscript{71} Both manganese and gadolinium, however, are toxic to humans as free ions. Both Gd-DTPA and MnCl\textsubscript{2} resulted in adverse but short-lived hemodynamic effects when administered intravenously to dogs.\textsuperscript{72} Gd\textsuperscript{3+} and Mn\textsuperscript{2+} have different biological effects, so the choice of metal ion must be specific to each application. Gd-DTPA, for example, can cause nephrogenic systemic fibrosis (NSF), a rare but potentially lethal disorder, in patients with kidney disease.\textsuperscript{57} Chronic manganese exposure, on the other hand, can cause a serious neurological disorder, thus necessitating careful regulation of dose.\textsuperscript{73–75} Gd\textsuperscript{3+} is two orders of magnitude more toxic to certain aquatic microbial communities than Mn\textsuperscript{2+}.\textsuperscript{76} Since all administered drugs are eventually excreted from the body, ecological effects are a relevant consideration when designing MRI CAs.

In short, the aim is to create a new carbon nanotube-based MRI CA by incorporating Mn\textsuperscript{2+} ions into (or onto) US-tubes for several reasons. First, the aqueous chemistry of Mn\textsuperscript{2+} may actually enhance performance above that of GNTs by providing more sites for water coordination at biological pH levels. Secondly, a comparison between Mn\textsuperscript{2+}@US-tubes (manganonanotubes, MNTs), Mn\textsuperscript{2+}/Gd\textsuperscript{3+}@US-tubes (manganogadonanotubes, MGTs), and Gd\textsuperscript{3+}@US-tubes (GNTs) could reveal additional fundamental knowledge about the contributions of US-tubes to relaxivity. Third, Mn\textsuperscript{2+} agents offer unique biological interactions, thereby revealing structural information that
cannot be provided by Gd\(^{3+}\)-based CAs. Finally, Mn\(^{2+}\) appears to be less toxic than Gd\(^{3+}\), which is a certain advantage.\(^{71}\)

3.2. Methods

3.2.1. ICP digestion and sample preparation

In order to evaluate the effectiveness of various digestion methods for inductively-coupled plasma (ICP) analysis, undigested standard solutions of various metals were compared to digested samples of known volumes and concentrations. Metal standard solutions were heated to dryness in glass scintillation vials on a hot plate. Measured volumes of the digestion acid or oxidant were deposited and heated to dryness. The digestion matrix was added 2-3 times. Samples were diluted in 2% HNO\(_3\) and analyzed by ICP-optical emission spectroscopy (OES) or ICP-mass spectrometry (MS). Undigested samples were similarly diluted in 2% HNO\(_3\) and analyzed. For percent recovery experiments, 1000 mg/L metal standards in 2% HNO\(_3\) (Fluka) were digested and resuspended in 2% HNO\(_3\) to 5 mg/L concentrations and compared to undigested standards of the same quantity.

3.2.2. Mn\(^{2+}\)- and Gd\(^{3+}\)-loading of US-tubes

Mn\(^{2+}\) and Gd\(^{3+}\) ions were loaded into US-tubes in a similar fashion to the method described for GNTs in Chapter 2. Full-length single-walled carbon nanotubes produced via the arc discharge method (Carbon Solutions) were fluorinated and pyrolyzed to produce US-tubes.\(^{38}\) The US-tubes were purified with 12 M HCl to remove catalytic impurities, sonicated with Na metal in THF to debundle the tubes, and refluxed in 6 M
HNO$_3$ for five min, with DI water washing over a glass frit between each step.

Approximately 1 mg/mL of US-tubes were bath sonicated in a solution of GdCl$_3$·6H$_2$O or MnCl$_2$·4H$_2$O for 1 h and then washed with DI water over a glass frit to remove unbound ions. Samples were oven-dried and suspended in 0.17% (w/v) Pluronic® F 108NF Prill Poloxamer 338 (BASF) at 1 mg/mL. The sample was spun down at 3200 rpm, collecting the supernatant to remove unsuspended tubes.

### 3.2.3. Relaxivity analysis

Relaxation properties were measured using a Bruker Minispec (mq 60) benchtop relaxometer operating at 60 MHz and 37 °C. The longitudinal ($T_1$) relaxation times were obtained using inversion recovery pulse sequence. Sample concentrations were measured by ICP-OES. Relaxivity values were calculated according to Equation 2.1.

### 3.2.4. MRI analysis

Both $T_1$ and $T_2$ were measured on a clinical 1.5 T/3.0 T scanner (Achieva /Ingenia, Philips Healthcare, Best, The Netherlands). The spatial resolution for all the scans was 1.56 x 1.5 mm and the slice thickness was 3 mm. In the $T_1$ measurement, spin echo (SE) sequence with repetition time (TR)/echo time (TE): 6000/8.6ms (1.5 T), 9.5/6000 ms (3.0 T) and inversion time (TI): 50, 100, 200, 400, 800, 1000, 1200, 1400, 2000, 2500, 3000, 3500ms (1.5 T), 20, 50, 100, 200, 400, 800, 1000, 1200, 1400, 2000, 2500, 3000, 3500 ms (3.0 T) were used. In the $T_2$ measurement, turbo spin echo (TSE) sequence with TR/TE/ΔTE: 6000/20/20 ms and 20 echoes per TR were used for both 1.5 T and 3.0 T. Results for $T_1$ measurements taken at 1.5 T are reported and discussed.
3.2.5. Saline and serum challenge study

Dry, 3 mg samples of MNTs, MGTs, and GNTs were suspended in 4 mL of challenge medium, either phosphate-buffered saline (PBS, Sigma P5493, Ca- and Mg-free, 10 mM phosphate, 0.154 M NaCl) or 10% (v/v) fetal bovine serum (FBS) dissolved in PBS. Samples were probe-sonicated for in pulses 5 min total, 1 s on, 2 s off, then kept in a 37 °C water bath overnight. Samples were centrifuged through Amicon Ultracel 100K centrifugal filter units (Millipore) at 3200 rpm for 20 min. US-tubes remained in the filter, while challenge medium and any leaked ions eluted in the filtrate. Challenge medium was collected. Serum samples were digested, and both sets of samples were analyzed by ICP-OES as described above. For analysis of the second rinse, an additional 2 mL challenge medium was added to the previously challenged tubes and spun down as previously. Rinse solutions were analyzed for Mn$^{2+}$ and Gd$^{3+}$ content by ICP-OES.

3.3. Results and Discussion

3.3.1. ICP digestion techniques

In initial studies, attempts to load Mn$^{2+}$ into US-tubes, either with or without Gd$^{3+}$ ions also co-loaded in various ratios appeared to fail (Figure 3.1a, loading parameters outlined in Table 3.1). No sign of Mn$^{2+}$ remained in the tubes by ICP-OES or ICP-MS analysis, and washing data (Figure 3.1b and c) suggested that Mn$^{2+}$ had leaked out of the US-tubes. In the sample containing Mn$^{2+}$ only, the leakage appeared the most significant, with a steady release from the US-tubes at each washing (Figure 3.1c). This suggested that perhaps the Mn$^{2+}$ was forming bonds in equilibrium with some moieties (likely
carboxylates) on the sidewalls of the US-tubes, eventually being washed away completely with each fresh addition of DI water. A lack of Mn$^{2+}$ in the washes for the other Gd:Mn samples (1:1 and 3:7 Gd:Mn), however, indicated that perhaps the Mn$^{2+}$ had actually loaded, but was somehow not being detected by ICP.

Table 3.1 Loading parameters for GNTs, MGTs, and MNTs, Figure 3.1.

<table>
<thead>
<tr>
<th>Sample (Gd:Mn volume ratio during loading)</th>
<th>[Gd$^{3+}$] loading (mM)</th>
<th>[Mn$^{2+}$] loading (mM)</th>
</tr>
</thead>
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<td>Empty</td>
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<td>0.0</td>
</tr>
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</tr>
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</tr>
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</tr>
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<td>0:1</td>
<td>0.0</td>
<td>1.0</td>
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Figure 3.1 Initial metal loading and washing data for attempts to load US-tubes with Mn$^{2+}$ and/or Gd$^{3+}$ ions. (a) Percent metal loaded into US-tubes by weight. Samples were digested with HClO$_3$ prior to ICP analysis. (b) Metal content in DI water washes after metal ion loading was attempted. No acid digestion step was performed prior to analysis. Data points at “wash 0” indicate the concentration of the original loading solution, as outlined in Table 3.1. (c) Data identical to (b) except with “wash 0” omitted to view low concentrations at later washes.
For a typical ICP measurement, organic content must first be digested because it can clog the ICP instrumentation or damage the detector. As described in Section 3.2.1 above, US-tube samples are prepared for ICP analysis by oxidizing the carbon cage, which evaporates away as CO$_2$, leaving metal content in the scintillation vial. Contents of the vial are suspended in 2% HNO$_3$, which is a common matrix used for ICP measurements. Chloric acid (HClO$_3$) is a powerful oxidizing agent that can fully oxidize US-tube samples upon heating within minutes. This simple method does not interfere with Gd$^{3+}$ analysis, but was found to drastically reduce Mn$^{2+}$ recovery. This was discovered upon trace metal analysis by ICP of HClO$_3$-digested standards of known concentrations of Gd$^{3+}$ and Mn$^{2+}$ compared with their undigested counterparts (Figure 3.2). While Gd$^{3+}$ exhibited 99% recovery by this method, Mn$^{2+}$ had an abysmal 22% recovery rate. This result may be accounted for by the different chemical reactivities of aqueous Gd$^{3+}$ versus Mn$^{2+}$ ions and the solubility of their oxidation products. Even when oxidized to Gd$_2$O$_3$, Gd remains in oxidation state III and is readily dissolved in a variety of acids, including HCl and HNO$_3$.\(^{77-79}\) It is likely to do the same in the presence of HClO$_3$, which, in our system, can readily oxidize the US-tubes, leaving an acid-soluble Gd$^{3+}$ product behind. MnCl$_2$ likely oxidizes to MnO$_2$ (oxidation state IV) when heated in HClO$_3$, due to the formation of a brown precipitate that is characteristic of MnO$_2$. Manganese dioxide is insoluble in 2% HNO$_3$, thus explaining the low recoveries with the HClO$_3$ digestion.
In order to find a more suitable digestion method for Mn$^{2+}$, standard solutions of Gd$^{3+}$, Mn$^{2+}$ and other metal ions were digested with various acid/oxidant/reducer combinations. HNO$_3$ was chosen as an alternative to HClO$_3$ due to its common use as an oxidant for carbon nanotubes. H$_2$O$_2$ was added to attempt to increase the oxidizing action, and HCl was added to potentially reduce the insoluble MnO$_2$ back to water-soluble MnCl$_2$. As seen in Figure 3.2 the best digestion methods that were tested for Mn$^{2+}$ were both HNO$_3$ alone and HNO$_3$ followed by HCl. The explanation for success is the probable formation of Mn(NO$_3$)$_2$, a water- and acid-soluble salt. The reduced recovery for the method using H$_2$O$_2$ further corroborates the theory that strong oxidizing agents form an insoluble MnO$_2$ product, thereby reducing recovery. Adding HCl after HNO$_3$ turned out to be redundant in the case of Mn$^{2+}$ but improved recovery for Gd$^{3+}$. Digestion by HNO$_3$ or HNO$_3$ plus HCl was used for all subsequent studies to accurately assess Mn$^{2+}$ content in US-tube samples. The major drawback is the drastically increased digestion

Figure 3.2 Percent recovery of Gd and Mn standards by ICP-OES after digestion with various acid/oxidizing agent/reducing agent combinations.
times and volumes of liquid required for HNO$_3$ to fully oxidize the US-tubes. HClO$_3$ continued to be used as the method of choice for Gd$^{3+}$ analysis.

In the process of the ICP digestion recovery study for Gd$^{3+}$ and Mn$^{2+}$, recoveries for other metal ions were also tested. Fe$^{3+}$ exhibited 105% recovery with an HClO$_3$ digestion protocol, and La$^{3+}$ and Lu$^{3+}$ had strong results of 93 and 98%, respectively (Figure 3.3a). Strong recoveries for Gd$^{3+}$, Mn$^{2+}$, La$^{3+}$, and Lu$^{3+}$ were observed with a HNO$_3$ followed by HCl procedure (Figure 3.3b).
Figure 3.3 ICP digestion recoveries for Gd, Mn, La, Lu, and Fe by (a) HClO₃ and (b) HNO₃ followed by HCl (Fe not measured).

In addition to testing oxidizing and reducing agent combinations, a 1% (v/v) Spectrasol® solution was assessed as an alternative matrix to 2% HNO₃. Spectrasol® is a commercial surfactant solution comprised of octyl decyl dimethyl ammonium chloride, dioctyl dimethyl ammonium chloride, didecyl dimethyl ammonium chloride, and alkyl (50% C14, 40% C12, 10% C16) dimethyl benzyl ammonium chloride, as well as various inactive ingredients (Neogen). It was not found to substantially improve the recovery
(and therefore solubility) of the digestion products in any case, and hindered it in some (Figure 3.4).

![Graph showing percent recoveries for Gd\(^{3+}\) and Mn\(^{2+}\) standards by ICP analysis, using different digestion protocols and sample matrices of either 2% HNO\(_3\) or 1% (v/v) Spectrasol®.]

Figure 3.4 Percent recoveries for Gd\(^{3+}\) and Mn\(^{2+}\) standards by ICP analysis, using different digestion protocols and sample matrices of either 2% HNO\(_3\) or 1% (v/v) Spectrasol®.

### 3.3.2. Gd\(^{3+}\) ions preferentially load in US-tubes over Mn\(^{2+}\) ions

Once adequate trace metal analysis methods were established for Mn\(^{2+}\), treated US-tubes were again soaked with solutions of GdCl\(_3\) and MnCl\(_2\). It has been established that, in the case of loading Cu\(^{2+}\) into US-tubes, Cu\(^{2+}\) ions leak out unless co-loaded with Gd\(^{3+}\) ions.\(^{82}\) Thus, initial attempts to load Mn\(^{2+}\) into US-tubes focused on co-loading with Gd\(^{3+}\) ions. Results yielded a Gd:Mn molar ratio in the tubes of 25:1 (results not shown), with a negligible Mn\(^{2+}\) concentration detected (0.01 mM versus 0.25 mM for Gd). This was despite the fact that the MnCl\(_2\) concentration (90 mM) in the loading solution was 9 times higher than the GdCl\(_3\) concentration (10 mM). This clearly indicated that Gd\(^{3+}\) ions
preferentially load in US-tubes over Mn$^{2+}$ ions. This preference is indubitably the result of the different aqueous chemistry of Gd$^{3+}$ and Mn$^{2+}$ described in Section 3.1. While Gd$^{3+}$ precipitates as a hydroxyl-bridged cluster in the US-tubes, Mn$^{2+}$ remains water-soluble at neutral pH.

3.3.3. Mn$^{2+}$ can be loaded into US-tubes in the presence or absence of Gd$^{3+}$ ions

In order to circumvent or possibly take advantage of these chemical differences, absolute loading concentrations of GdCl$_3$ were reduced to avoid overloading the US-tubes with Gd$^{3+}$. Although Mn$^{2+}$ is still soluble at neutral pH, it is known to coordinate with carboxylates in a variety of systems.$^{83,84}$ Due to the treatment of US-tubes with 6 M HNO$_3$ prior to the loading step, carboxylate functional groups are likely abundant along the carbon cage,$^{56,57}$ particularly at the defect sites that are produced during US-tube preparation. Alternatively, we speculated that the formation of Gd$^{3+}$ ion clusters might physically or chemically trap Mn$^{2+}$ in the tubes as well. With this in mind, US-tubes were soaked with the GdCl$_3$ solutions at a concentration of 0.05 mM, nearly two orders of magnitude lower than previous attempts, while the MnCl$_2$ concentrations were varied from 0 mM to 5 mM. With the decreased GdCl$_3$ loading concentration, Mn$^{2+}$ ions were able to associate with the US-tubes, up to a weight percent of almost 1% (Figure 3.5a). Gd$^{3+}$ loading remained nearly constant around 0.6%. Interestingly, a MnCl$_2$ loading concentration ten times higher than that of GdCl$_3$ was required to achieve an approximate 1:1 weight percent ratio with Gd$^{3+}$ in the US-tubes, and even with an initial concentration 100 times higher, Mn$^{2+}$ still only modestly out-loaded Gd$^{3+}$ ions (Figure 3.5b). Nevertheless, a hybrid MGT synthesis was achieved under these new conditions.
Figure 3.5 Percent of metal content loaded into US-tubes by weight. (a) Weight percent Gd$^{3+}$ and Mn$^{2+}$@US-tubes as a function of Mn$^{2+}$ loading concentration, with the Gd loading concentration fixed at 0.05 mM. (b) Weight percent expressed in relation to Gd:Mn molar ratio during synthesis.
3.3.4. MGTs have high relaxivities

In order to assess the potential of these new Gd/Mn hybrid carbon nanomaterials as an MRI contrast agent, relaxivities were measured for the MGTs (Figure 3.6). The first thing to note is that the MGTs measured had \( r_1 \) values ranging from 40 – 80 mM\(^{-1}\)s\(^{-1}\), which is remarkable for any \( T_1 \)-weighted MRI CA (see Table 2.1 as well as discussion below for comparison) (Figure 3.6a). However, as the Mn:Gd molar ratio in the MGTs increases, the \( r_1 \) values decrease, possibly in a linear fashion (Figure 3.6b). This is not surprising, given that an aqueous GdCl\(_3\) solution had a measured \( r_1 \) value (10 mM\(^{-1}\)s\(^{-1}\)) 67\% higher than that of MnCl\(_2\) (6 mM\(^{-1}\)s\(^{-1}\)). However, the results did run counter to the initial hypothesis, that in the US-tubes, Mn\(^{2+}\) could have an advantage over Gd\(^{3+}\) in having more potential sites available for water coordination at a biological pH. Both the results given here and in Chapter 2 indicate that (1) GNTs already have a high \( Q \) value, from a minimum of two\(^{44}\) or three\(^{28}\) to a maximum of six\(^{29}\) and (2) the inclusion of Mn\(^{2+}\) offers no additional relaxivity boost on a per-ion basis. In addition to having a lower relaxivity than Gd\(^{3+}\), Mn\(^{2+}\) ions may also sacrifice water coordination sites in the process of coordinating to the US-tubes, which they have been demonstrated to do in the presence of carbonates, going from \( Q = 6 \) to \( Q = 3 \) on binding.\(^{85}\)
Figure 3.6 Relaxivity ($r_1$) data for MGTs at 37 °C, 1.41 T, 60 MHz. (a) Relaxivity as a function of MnCl$_2$ loading concentration, with Gd loading concentrations constant at 0.05 mM. (b) Relaxivity data for the same MGTs, plotted as a function of Mn:Gd molar ratio after loading into US-tubes.
Encouraged by the loading of Mn$^{2+}$ ions into GNTs, Mn$^{2+}$ was loaded into US-tubes in the absence of Gd$^{3+}$ ions. These MNTs exhibited a Mn$^{2+}$ weight percent of 0.7% (Figure 3.7). MGTs contained 0.5% (w/w) Gd and Mn each. GNTs achieved greater loading, with a weight percent of 2.8%.

![Graph showing metal content loaded into US-tubes by weight](image)

Figure 3.7 Percent of metal content loaded into US-tubes by weight, a comparison of MGTs, MNTs, and GNTs. Loading concentrations were 0.05 mM Gd and 0.5 mM Mn for MGTs, 0.5 mM Mn for MNTs, and 0.5 mM Gd for GNTs.

### 3.3.5. Mn$^{2+}$ leaks from US-tubes when biologically challenged

To test and compare MGTs, MNTs, and GNTs for biological applications, the samples were challenged with solutions of phosphate buffered serum (PBS) and 10% (v/v) fetal bovine serum (FBS) in PBS, after being washed 20 times with DI water as part of the synthesis process. While the DI water washes failed to remove Mn$^{2+}$ or Gd$^{3+}$ ions from US-tubes after an initial excess of free ions had been lost (Figure 3.8), the biological challenge removed significant quantities of Mn$^{2+}$ from both the MGTs and MNTs,
whereas no Gd\(^{3+}\) leaked from the MGTs or GNTs (Figure 3.9). For comparison, Cu\(^{2+}\) has been shown to leak from US-tubes, but not from GNTs.\(^{82}\) The difference between biological challenge results for Cu\(^{2+}\) and Mn\(^{2+}\) has two possible explanations. First, the synthesis for Cu\(^{2+}\)@GNTs employed NaOH to adjust the pH to neutral, whereas the MNT and MGT syntheses only used DI water washes to raise the pH to about 5.5. This extra step could have assured the formation of Cu(OH)\(_2\) or hydroxy-bridged Cu\(^{2+}\)/Gd\(^{2+}\) clusters in the US-tubes. Secondly, Cu\(^{2+}\) is inherently less soluble than Mn\(^{2+}\) at neutral pH. The k\(_{sp}\) values for Gd(OH)\(_3\), Mn(OH)\(_2\), and Cu(OH)\(_2\) are 1.8×10\(^{-23}\), 2.1×10\(^{-13}\), and 1.6×10\(^{-19}\), respectively.

![Figure 3.8](image.png)

Figure 3.8 Mn\(^{2+}\) or Gd\(^{3+}\) leakage from US-tubes washed with DI water after loading, measured by ICP.
Figure 3.9 Leakage of Mn$^{2+}$ or Gd$^{3+}$ ions from loaded and washed US-tubes upon challenge with (a) PBS and (b) 10% FBS in PBS.
These results once again highlight the differences between Mn$^{2+}$ and Gd$^{3+}$ chemistry. Whereas Gd$^{3+}$ forms hydrophobic (and thus carbon nanotube-philic) complexes at neutral pH, the Mn$^{2+}$ ions associated with the US-tubes form only moderate associations with the carboxylate groups on the US-tube surface. In the presence of PBS, the Mn$^{2+}$ ions likely achieve equilibrium with the Cl$^-$ and PO$_4^{3-}$ anions and the Mn$^{2+}$-carboxylate complex, thus washing a portion away with every subsequent challenge. Since no significant differences in Mn$^{2+}$ loss were seen between the PBS and serum challenge (paired t-test, p = 0.1990), the salt presence is probably the dominant effect. Serum proteins may theoretically contribute by forming complexes with Mn$^{2+}$ as they do in the nature.\textsuperscript{86}

Ion leakage under biological conditions is not necessarily a disadvantage for all clinical applications. With a slow, steady release from the US-tubes, Mn$^{2+}$ may exchange with other metals present in the body, such as Zn$^{2+}$ exchange in the liver in the case of FDA-approved Mn-DPDP.\textsuperscript{66} Alternatively, the toxicity of Mn$^{2+}$ could be exploited as a drug. The carbon cage of the US-tubes provides a scaffold for targeting moieties, and the Mn$^{2+}$ release would occur predominantly in the chosen target. Inherent MRI contrast of the agent could simultaneously be used to track the drug \textit{in vivo}.

### 3.3.6. Relaxivity comparison of MNTs, MGTs, and GNTs

The prepared MNTs, MGTs, and GNTs were compared in terms of $r_1$ normalized by total ion concentration ([Mn$^{2+}$]+[Gd$^{3+}$] in the case of MGTs) (Figure 3.10). GNTs had a relaxivity (110 mM$^{-1}$s$^{-1}$) about 60% higher than MNTs (65 mM$^{-1}$s$^{-1}$) or MGTs (74 mM$^{-1}$s$^{-1}$). This enhancement correlates well with the 67% increase in $r_1$ for GdCl$_3$ over MnCl$_2$. 
In this study, the US-tubes alone made a substantial contribution to the relaxivity performance. MNTs have $r_1$ 12 times higher than MnCl$_2$, which is essentially the free Mn$^{2+}$ ion in aqueous solution and 23 times higher than the clinical agent Mn-DPDP (2.8 mM$^{-1}$s$^{-1}$). This result demonstrates the universality of the US-tube as an MRI performance enhancement agent. For the data shown, $r_1$ is calculated based on the differences in contrast between the CA and DI water. However, if $r_1$ is based on the difference in contrast between the CA and US-tubes suspended in Pluronic® surfactant solution, the results are 94, 50, and 59 mM$^{-1}$s$^{-1}$ for GNTs, MNTs, and MGTs, respectively. These slight deviations from the original values indicate that the US-tubes alone do not contribute the bulk of the contrast enhancement over the free ions. Rather, the US-tubes provide a unique, confining environment, which, when paired with paramagnetic ions, creates a synergistic effect, thereby boosting relaxivity for all three agents by about 13-fold. It is likely that at least part of this enhancement comes from the US-tubes forcing short metal-proton distances for metal-coordinated water molecules, as evidenced by the short Gd-O (and therefore short Gd-H) distances measured by X-ray absorptions spectroscopy for the GNTs. Another probable and important contribution in the metal-US-tube hybrid system is the increased tumbling time afforded by the bulky US-tubes, as discussed for GNTs in Chapter 2.
3.3.7. MRI phantoms of Mn$^{2+}$-based US-tube CAs

The quantitative results outlined above were visualized in the MRI phantoms of the MRI CA suspensions \textit{ex vivo} (Figure 3.11). The brighter contrast for GNTs, MGTs, and MNTs is readily apparent in comparison to all controls, including water, US-tubes suspended in Pluronic®, Pluronic® solution, and the aqueous Gd$^{3+}$ and Mn$^{2+}$ chloride solutions used for loading of the US-tubes.
Figure 3.11 $T_1$-weighted MRI phantoms taken at 1.5 T of MGTs, MNTs, and GNTs suspended in equal total metal ion concentrations in 0.17% (w/v) Pluronic® solutions. US-tubes suspended in Pluronic®, DI water, Pluronic solution®, and aqueous solutions of the loading Gd$^{3+}$ and Mn$^{2+}$ chlorides are shown for comparison. TI from left to right in each panel is 100, 200, 400, and 800 ms.

3.4. Conclusions

This work has culminated in the development of two new carbon nanotube-based systems with potential for clinical application as MRI CAs: manganonanotubes and manganogadonanotubes. Neither upheld the original hypothesis that the solubility of Mn$^{2+}$ at neutral pH could increase available sites for water coordination, and thereby increase the relaxivity above that of GNTs. However, MNTs and MGTs exhibited high MRI performance, with $r_1$ of 65 and 74 mM$^{-1}$s$^{-1}$, respectively. These results revealed the unique and universal applicability of US-tubes as MRI CA-enhancement agent. Although Mn$^{2+}$ ions leaked from US-tubes when challenged with PBS or serum, this does not preclude their use as a clinical MRI CAs. With dose regulation, the leakage could be desirable in certain conditions, as is the case with the FDA-approved Mn-DPDP. MNTs offer a high-performance alternative where Gd-based agents are undesirable or where Mn$^{2+}$ biochemistry is advantageous.
Chapter 4

Magnetic isolation of quiescent breast cancer cells

4.1. Introduction

Cells in a typical cell cycle undergo several phases. In Gap 1 phase (G1), cells increase in size and prepare for DNA synthesis. Next, DNA replication occurs during the synthesis (S) phase. During Gap 2 (G2), cells continue to grow and prepare for mitosis (M), or cell division. Daughter cells then resume the pattern, continuing the cycle. If stimulated by the environment, cells can also leave the cell cycle and enter a resting phase, called Gap 0 (G0). This arrest can be reversible, defined as quiescence, or irreversible, defined as senescence. Cellular quiescence plays a crucial role in biological function. By entering and exiting the cell cycle, quiescent cells can live longer and avoid accruing mutations that can occur over many divisions. This is of particular importance in stem cell biology, where the stem cells must maintain genetic integrity for long periods of time.
Despite the significance of cellular quiescence, much remains unknown. For example, whether quiescence is a static or dynamic state. In order to learn more about cell dormancy, quiescent cells must first be isolated. However, this task is far from trivial. Although the ability to exit the cell cycle is a common feature of various tissue types, quiescent cells prove to be elusive targets. A major reason for this is that their definition is based on a lack of cell division, rather than having any distinct physical traits. No clear, universal biomarkers can distinguish them for isolation. Stem and progenitor cells that have the ability to remain quiescent for long periods of time usually constitute small fractions of tissue, creating a need for sensitive methods of detection and isolation.

4.2. Background

Several techniques including flow cytometry, centrifugation, and magnetic separation have been previously utilized for the isolation of quiescent cells. Each of these has its benefits and limitations as described below.

4.2.1. Flow cytometry

Flow cytometry is a powerful technique for analyzing and sorting cells from single-cell suspensions. In flow cytometry, particulate suspension is injected into a stream of fluid through a laser beam. The sample scatters or emits light, according to its morphological properties and the presence or absence of fluorophores on the cell. Cells are then sorted according to the user’s chosen parameters by electrostatic charging of selected cell types. Once charged, the cells exhibiting the desired optical properties are deflected into a sample receptacle. Cell sorting by this method is remarkably versatile and
can isolate rare cell populations with known biomarkers. However, flow cytometry has several drawbacks. First, it requires samples to be prepared as single-cell suspensions, as opposed to being able to study bulk tissue samples or cell behavior in vivo. Secondly, flow cytometric cell sorting is both cumbersome and expensive, requiring advanced instrumentation and training. Most importantly, the sorting process can alter cell properties, specifically membrane integrity, which can likely skew cell behavior post-sort.\textsuperscript{91}

4.2.1.1. Biomarkers

A common detection and sorting method to purify quiescent cells is the use of DNA-binding fluorophores, such as Hoescht 33342 or propidium iodide (PI). For Hoescht and other DNA labels, cell populations with typical cell cycle distributions will exhibit a specific pattern of fluorescence intensities. Once debris and cell clusters, which can cause interfering light scattering, are excluded from flow cytometric analysis, cells that have entered G2 phase or mitosis have exactly twice as many copies of DNA as cells in G1 or G0, which have yet to begin S-phase. Thus, cells with fluorescently-labeled DNA that are in G2 have double the fluorescence intensities of cells in G0/G1. An example of an established protocol exploits the A-T base pair fluorescent label Hoechst 33342 combined with RNA binding dye Pyronin Y. The RNA label allows one to distinguish between quiescent and senescent cells, or those that are not actively dividing but are producing protein from messenger RNA (mRNA). Low intensities of both dyes indicate cells in G0, whereas low Hoescht and high Pyronin indicates senescence (G1).\textsuperscript{92}
In addition to DNA labeling, other cellular features can be exploited to probe cell cycle status by flow cytometry. The gene Ki67 is expressed in proliferating cells. By designing a green fluorescent protein (GFP) reporter of the gene, proliferating cells can be distinguished from their non-dividing counterparts.\textsuperscript{93} In another study, a new cell cycle indicator was developed to identify quiescent cells by flow cytometry.\textsuperscript{94} Exploiting higher levels of cyclin-dependent kinase inhibitor p27 in quiescent cells, the fluorescent probe was able to distinguish between G0 and G1 cells. Other strategies using biomarkers to identify slow-cycling populations, include the expression of tyrosine kinase Tie2, which identifies quiescent hematopoietic cells.\textsuperscript{95}

Although incredibly valuable for specific cell types with known gene expression profiles, the use of biomarkers to identify or isolate quiescent cells is inherently indirect. Gene expression is specific to each cell type and microenvironment, and cannot be universally applied.

\textbf{4.2.1.2. Label retention}

By exploiting label retention in non-dividing cells, quiescence can be identified through function, rather than expression of a particular protein.\textsuperscript{96} Label retention techniques include the use of thymidine analogues, such as bromodeoxyuridine (BrdU), tritiated thymidine, and 5-ethyl-2'-deoxyuridine (EdU), and tritiated thymidine, which incorporate into the DNA of dividing cells during S-phase and can later be detected and isolated by immunostaining, direct fluorescence labeling, or liquid scintillation counting. However, these methods require cell fixation and permeabilization, rendering the quiescent population dead, and thus unfit for further functional assays.
As an alternative, retention of live fluorescent dyes, such as EGFP-tagged histone 2B (2B-GFP), PKH26, and carboxyfluorescein diacetate, succinimidyl ester (CFSE) can be used to isolate a slow-cycling cells. 2B-GFP targets chromatin, PKH26 is a lipophilic molecule that adheres to the cell membrane, and CFSE bonds to amides within the cytoplasm. Unlike BrdU or Edu, PKH26 and CFSE can be fluorescently imaged on living cells or tissue.

Label retention is advantageous in that it relies only on cellular function, or lack of division, to isolate quiescent cell populations. Until now, however, it has always been used in tandem with flow cytometry, which has drawbacks as discussed above. One possible flaw is the presumption that the label retaining cells are quiescent. Though a likely explanation, relative “retention” could also be explained by initial non-uniform cell labeling, resulting in uneven distribution of dye, or by increased uptake (or decreased efflux) of dye in certain cells. It may also fail to distinguish between slow-cycling cells and terminally differentiated cells since, in both cases, cells are not dividing or losing label. In the latter case, however, the cell cycle arrest is permanent, and not reversible, like quiescence. Additionally, stem cells have been shown to rapidly efflux dyes more efficiently than other cell types, which could potentially render label retention with dyes to be counter-productive in identifying stem-like cell populations. Finally, fluorescent dyes have varied lifetimes, depending on the cell type being labeled as well as the robustness against photobleaching (dye deterioration due to light exposure), posing a potential setback for long-term quiescent tracking studies.
4.2.2. Density-gradient centrifugation

Certain cell types lend themselves to isolation methods that do not require flow cytometry, but exploit physical traits unique to quiescent cells within a population. Slow cycling yeast cells, for example, have been separated from their rapidly dividing counterparts by density gradient centrifugation.\textsuperscript{104} This method relied on the inherent size differences between the two cell types in yeast, a distinction that does not transcend other cell types.

4.2.3. Magnetic cell separation

Magnetic cells separation by magnetic activated cell sorting (MACS)\textsuperscript{105} or DynaBeads\textregistered\ has been used to isolate cells on the basis of known biomarker expression, similar to the flow cytometric methods discussed above. However, they likewise rely on biomarkers that are not present in all cell types and can have ambiguous expression. In order to develop a relatively inexpensive, facile, and function-based method to isolate quiescent cells, we combined principles of label retention with the gentle, readily accessible, bench-top cell separation method of MACS\textsuperscript{®} (Miltenyi). Our hypothesis was that cells which could retain an internalized magnetic label over time would (1) represent a relatively quiescent fraction of the cell population and (2) be captured on a reversibly magnetized column. In order to accomplish this task, two magnetic labels were tested: gadonanotubes (GNTs) and iron oxide nanoparticles (IONs). The general scheme is outlined in Figure 4.1.
Two magnetic labels were tested for the proposed scheme: GNTs and iron oxide nanoparticles (IONs). With nanoscale dimensions, biocompatible surfactant coatings, and a lipophilic carbon cage, GNTs were chosen for their established ability to pass through cell membranes, their demonstrated ability to act as magnetic labels for GNT-labeled cell retention in heart tissue, and their advantageous properties as MRI contrast agents. Dextran-coated Molday ION(-)™ particles were also selected because their close structural relative Feridex I.V.® was FDA approved for use in humans as an MRI contrast agent, their superparamagnetic iron oxide cores would likely enable cell manipulation by external magnetic fields, and there were established protocols for cellular internalization assisted by transfection agent poly-L-lysine.
4.3. Methods

4.3.1. Cell models

Four breast cancer cell types were employed for these studies. Initial testing began with MC1 cells, a xenograft tumor line derived from pleural effusion of metastatic breast cancer. Until this work, MC1 cells are propagated exclusively in vivo in immunodeficient animal hosts, providing access to primary tumor cells. To prepare dissected MC1 tumors for in vitro studies, they were minced digested into single cell suspensions with collagenase type III for 3 h at 37 °C, by a previously reported method.\(^{110}\) Later studies used SUM159, LM2, and MDA-MB-231 cell lines due to their ease of in vitro manipulation and low expression of the claudin protein, since this breast cancer subtype was found to be enriched for stem-like cell content.\(^{111-113}\)

4.3.2. Cell culture

Mammary Epithelial Cell Growth Medium (MEGM\(^{TM}\), Lonza) was supplemented with final concentrations of 0.004 mg/mL heparin, 2×10\(^{-4}\) mg/mL Gibco\(^{®}\) EGF recombinant human protein (Life Technologies), 2×10\(^{-5}\) mg/mL Gibco\(^{®}\) fibroblast growth factor-basic (bFGF, Life Technologies), Gibco\(^{®}\) Antibiotic-Antimycotic (Life Technologies), and Gibco\(^{®}\) B-27\(^{®}\) serum-free supplement. This supplemented medium is referred to as MEGM+. MEGM+ with 0.5% (v/v) autoclaved methyl cellulose (Sigma) dissolved overnight on a warm hot plate with stirring was used in long-term cell incubation studies (Figure 4.11, Figure 4.12, and Figure 4.13) to promote the formation of individual mammospheres in suspension rather than clumps of cells that can form
without methyl cellulose present. For all studies involving the incubation of MC1 cells, MEGM+ was employed, and cells were exclusively propagated in suspension.

In order to propagate LM2 and SUM159 cells prior to magnetic labeling, frozen cultures were rapidly thawed in a 37 °C water bath and resuspended in fresh medium corresponding to the cell type, then incubated in a T-75 flask at 37 °C. For all studies, cells were pelleted by centrifugation at 300 rcf for 5 min at 4 °C. Medium was changed twice weekly until reaching confluence, at which stage the adherent cells were washed with phosphate-buffered saline (PBS), treated with 0.25% trypsin-EDTA for 5 min at 37 °C, and recovered in serum-containing medium to be collected for labeling. LM2 cell medium consisted of Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS and 1X Antibiotics-Antimycotic (AA). SUM159 cell medium consisted of Ham’s F12 Medium supplemented with 5 μg/mL insulin, 1 μg/mL hydrocortisone, 10 mM HEPES, 5% (v/v) FBS and 1% (v/v) penicillin-streptomycin.

4.3.3. Cell labeling with magnetic nanoparticles

Stock solutions of ~100 μM Gd Pluronic®-wrapped GNTs were prepared as described in Section 2.4.2. In order to sterilize GNTs, they were incubated at room temperature under UV lighting for 3 h prior to use in biological experiments. GNT labeling. MC1 or SUM159 cells were incubated at 37 °C overnight at a density of 10⁵ cells/mL with MEGM+ partially replaced with the GNT suspension to achieve concentrations ranging from 10-60 μM Gd.

For the GNT labeling concentration study, flasks were placed on an external magnet NdFeB magnet (K&J Magnetics) during incubation to potentially enhance
magnetization of GNT-labeled cells. The cells were washed with PBS, including an acid strip (50 mM glycine-HCl, 100 mM NaCl, and 2 mg/mL polyvinylpyrrolidone at pH 3.0) used in other studies to remove GNTs from the cell surface.

For iron oxide nanoparticle (ION) uptake, a stock solution of 10 mg Fe/mL carboxyl-terminated, dextran-conjugated Molday ION(-)™ particles (Biopal) was mixed with poly-L-lysine (BioPal) in a 3.3:1 volume ratio and diluted with cell culture grade water to 2 mg Fe/mL and incubated at room temperature for 1 h prior to cell labeling. The mixture was diluted to 10 μg Fe/mL with methylcellulose-free cell culture medium for all labeling studies, unless otherwise indicated. Cells were added to obtain a final concentration of 10^5 cells/mL and incubated in Corning ultra-low attachment flasks at 37 °C overnight, 12-24 h. Cells were centrifuged down at 300 rcf for 5 min at 4 °C and washed with 0.5-2 mL PBS, then plated in fresh medium for further studies.

4.3.4. Transmission electron microscopy (TEM)

Labeled cell pellets were resuspended in a modified Karnovsky's fixative in 0.1 M cacodylate buffer, pH 7.4 at room temperature for 1 h prior to mounting and sectioning for TEM. Cells were centrifuged down at 300 rcf for 5 min in 1.5 mL Eppendorf tubes, then dispersed in and maintained overnight at 4 °C over night in fixative. Cells were spun down as before and washed several times with PBS. The cells were osmicated for 30 min before dislodging the cell pellet and mincing into 1 mm cubes. The cubes of cells were returned to 20 mL glass vials and osmicated for another 30 min. After several rinses in high purity water, the cells were stained en bloc in saturated aqueous uranyl acetate for 45 min. Dehydration began with 30% EtOH and carried in increments through to 100%
EtOH. Cells were infiltrated with a progressively higher ratio of embedding resin to
EtOH, and then given 3 changes of pure resin for 3 h each. Cell cubes were embedded in
Spurr's Low Viscosity resin\textsuperscript{116} and polymerized at 65 °C overnight. Thin sections were
cut on a Diatome Ultra45 knife, using an RMC MT6000-XL ultramicrotome. The
sections were viewed on a Hitachi H7500 transmission electron microscope and
images were captures using a Gatan US1000 digital camera and Digital Micrograph,
v1.82.366 software.

4.3.5. Magnetic cell separation

Magnetic label-retaining cells were isolated from nonmagnetic cells using a
MACS\textsuperscript{®} apparatus fitted with an MS column (Miltenyi Biotec). Manufacturer’s
instructions were followed, with the exception of use of external magnetic beads. The
column was rinsed with PBS and 0.5-1 mL cell suspension ($\leq 10^5$ cells/mL) was
introduced into the column hoisted on a permanent magnet. The ferromagnetic beads
within the column are reversibly magnetized in the presence of the magnet. The column
was washed with 1 mL PBS to elute all nonmagnetic cells and then removed from the
magnet. Magnetic cells were forced through the column with a manual plunger. Cells in
each fraction were counted by Beckman Coulter Vi-CELL\textsuperscript{®}.

4.3.6. Fluorescence imaging

Unfixed LM2 or MC1 cells were labeled with IONs or GNTs, with flasks resting
on an external NdFeB magnet (for MC1 cells only) during overnight incubation at 37 °C,
and stained with green fluorescent CFSE. Unlabeled cells (those without GNTs or IONs)
were stained with red fluorescent PKH26. The two populations were combined and
subsequently separated by MACS®. Droplets of resulting fractions were pipetted onto glass slides within a circle drawn by a hydrophobic barrier pen. Slides were imaged on a Zeiss upright fluorescence wide field microscope.

**4.3.7. Cell tracking velocimetry (CTV) measurements**

CTV assay was performed at the Cleveland Clinic Lerner Research Institute, Department of Biomedical Engineering to assess the motion of the cells in a magnetic field, as previously described. The work described here used a specially designed magnet, referred to internally as “Mark IV”, capable of evaluating particles with high induced magnetic moments and imparting a constant velocity on each particle. A cell suspension was introduced into a 0.2 × 2.0 mm internal diameter glass channel by syringe. The magnetic motions of the magnetically-labeled cells were observed with a 5x objective on a custom assembled Olympus microscope (Olympus, Japan) and a Retiga 2000R CCD camera (Qimaging, Surrey, BC, Canada). Images captured by the camera were transferred to PC RAM and then saved to a PC hard drive. The camera was controlled by Video Savant 4 software (IO Industries, Ontario, Canada). The particles were flagged and tracked between successive frames with in-house designed software called ImagView. Further data processing was accomplished with EXCEL Macros to achieve mean cell velocities and associated statistics for hundreds to thousands of tracked cells. The fundamental magnetic properties of the cells were expressed in terms of magnetophoretic mobility \( m \), which is defined by Equation 4.1, where \( \Delta \chi \) is the difference between the magnetic susceptibilities of the cells and the fluid, \( V \) is the cell volume, \( \eta \) is the fluid viscosity (9×10^{-4} kg/m-s for these studies), and \( R \) is the cell radius (7.5 \( \mu \)m for these cells).
Equation 4.1 Definition of magnetophoretic mobility ($m$).

The measured cell velocities are related to $m$ according to Equation 4.2, where $B_0$ is the applied magnetic field intensity and $\mu_0$ is the magnetic permeability of free space.  

$$v = m\nabla \left( \frac{B_0^2}{2\mu_0} \right)$$

Equation 4.2 Relationship between cell velocity ($v$) and mobility ($m$).

4.3.8. Inductively-coupled plasma mass spectrometry (ICP-MS)

In order to determine the average iron content in labeled cells, samples of GNT- or ION-labeled MC1 or LM2 cells were digested in scintillation vials using several 0.5-1 mL additions of HClO$_3$ (for Gd analysis) or fuming HNO$_3$ (for Fe analysis) at 100 °C. Once dry, samples were dissolved in 2% HNO$_3$ then analyzed on a PerkinElmer ELAN® 9000 W ICP-MS system.

4.3.9. EdU uptake and flow cytometry analysis.

LM2 cells were ION-labeled and incubated as described in the cell culture methods section for one month to allow for cell division. MDA-MB-231 cells, from which LM2 cells are derived, were cultured adherently prior to MACS in order to provide a GFP negative control for flow cytometry. EdU dissolved in DMSO was added to flasks of nonadherent LM2 cells with culture medium for a final concentration of 8.3 μM EdU, 0.083% (v/v) DMSO, for 1 h at 37 °C. Flask contents were pelleted and 500 μL 0.05%
trypsin-EDTA was added to each pellet and incubated for 5 min in a 37 °C water bath. 3 mL LM2 medium was added to stop the trypsinization.

Coupling of a fluorescent dye to EdU-containing cell samples was achieved according to a modified version of manufacturer instructions (Click-iT® Alexa Fluor Imaging Kit, Life Technologies). Samples were pelleted and washed with 1 mL 1% (v/v) BSA in PBS then resuspended in 100 uL 10% neutral buffered formalin and incubated at room temperature in the dark for 15 min. Alexa Fluor® 647 (AF647) reaction cocktails were prepared by mixing 3.5 mL PBS, 80 uL CuSO4, 20 μL AF647 azide, 40 μL 10x additive, and 360 μL DI H2O. 500 μL of this suspension was added to each sample as well as an MDA-MB-231 EdU positive control. 500 μL PBS was substituted for unlabeled and DAPI-labeled MDA-MB-231 samples, and a GFP+ LM2 sample. These suspensions were incubated at room temperature in the dark for 30 min, then pelleted and resuspended in DAPI in 0.5% (v/v) Triton™ X-100 in PBS (or triton only for unlabeled, GFP, and EdU controls). Samples were separated (retaining a fraction of unsorted cells in each replicate) by MACS®, using DAPI/triton as an eluent. Unsorted samples were retained for comparison. All cell samples were filtered through a 35 μm filter into BD falcon tubes prior to analysis. Flow cytometric analysis of resulting fractions was performed on a BD LSRFortessa™ Cell Analyzer.
4.4. Results & Discussion

4.4.1. Cellular uptake of nanoparticles

Intracellular uptake of both GNTs and IONs by SUM159 and LM2 breast cancer cell types was confirmed by TEM (Figure 4.2 and Figure 4.3) and quantified by ICP-MS (Figure 4.4a and Figure 4.5a). Electron micrographs indicated efficient uptake of particles, likely via endocytosis, based on the engulfing of particles in vacuoles within the cytoplasm. On average, particle internalization reached a maximum of $\sim 3.7 \times 10^9$ Gd ions/cell for GNTs and $\sim 1.7 \times 10^6$ IONs/cell. Neither intracellular label adversely affected cell viability (Figure 4.4b and Figure 4.5b).

Figure 4.2 TEM images of uptake of IONs by LM2 cells over time. (a) Unlabeled LM2 cells. LM2 cells incubated in 10 μg Fe/mL ION suspension for (b) 1 h, (c) 2 h, (d) 4 h, and (e) 12 h. Scale bar 1 μm.
Figure 4.3 TEM images of magnetically labeled SUM159 breast cancer cells. (a) ION-labeled SUM159 cell undergoing chromosome condensation in preparation for mitosis. (b) IONs located within vacuoles and outside the cell membrane (red arrows) of a SUM159 cell, labeling concentration 15 μg Fe/mL. (c) GNT-labeled SUM159 cell, labeling concentration 30 μM Gd. (d) GNTs located within vacuoles of SUM159 cell, magnification of red box in (c).

High intracellular ION concentrations were readily visualized by TEM for both LM2 and SUM159 cells. By capturing a cell preparing for mitosis (Figure 4.3a), the portioning of IONs during division can be imagined. While not exactly symmetric, one can see that an abundance of particles should remain in each daughter cell after division is complete, based on the pattern of chromosome condensation and the location of ION-filled vacuoles in the cell. High concentrations of GNTs in SUM159 cells were not
observed by TEM. This can be accounted for in multiple ways. First, labeling is not uniform, as can be seen by the large standard errors by ICP (Figure 4.4a), especially at high Gd labeling concentrations. Secondly, individualized tubes would be difficult to distinguish from other intracellular features. Thus, only aggregated GNTs are observed. Furthermore, although an acid-wash buffer was used to remove external GNTs from the cell surface, ICP values still represent the combination of internalized GNTs as well as any remaining in the cell suspension after several washing steps. Another important consideration is that the cell type measured by ICP was not imaged by TEM, which could also account for the discrepancies in uptake.

![Figure 4.4](image)

**Figure 4.4** (a) GNT uptake in MC1 cells at various labeling concentrations. (b) Viability of GNT-labeled cells at various concentrations, as quantified according to trypan blue exclusion in viable cells. No significant decrease in viability was observed.
4.4.2. Effective cell capture by magnetic column

While a variety of commercial and custom methods have been employed to separate magnetized cells, the MACS® column was chosen due to widespread accessibility and use in biological research settings.\textsuperscript{118–121} However, the MACS® column is designed for retention of cells that have been immunolabeled with external magnetic beads ranging in diameter from 50 nm to 3 μm. In order to test whether the apparatus could be adapted to capture cells \textit{internally} labeled with 30 nm diameter particles with ~10 nm diameter iron oxide cores, GNT- (labeling concentration 30 μM Gd) and ION-labeled (labeling concentration 50 μg Fe/mL for MC1 and 10 μg Fe/mL for LM2) cells were stained green with the live cell fluorescent dye CFSE and mixed with red PKH26 dye-labeled nonmagnetic cells. Fractions were imaged by fluorescence microscopy (Figure 4.6) The column effectively separated the green, magnetic cells from the red, nonmagnetic cells, creating an ION-labeled fraction whose purity rivals or surpasses that

Figure 4.5 (a) Average ION uptake by LM2 cells as a function of labeling concentration. (b) Percentage trypan blue exclusion viability at various ION labeling concentrations. No significant loss in viability was observed.
of flow cytometry-sorted samples. Both MC1 and LM2 cells demonstrated the capability of using this method to isolate internally magnetized cells. Qualitatively, however, the majority of GNT labeled cells were not captured by this apparatus, as many green GNT-labeled cells eluted in the first “nonmagnetic” fraction, the eluate. However, any cells remaining magnetic in the second fraction, the retentate (captured on the magnetic column), were indeed from the GNT-labeled population. This result indicated that (1) the IONs have higher magnetic susceptibilities than GNTs and/or (2) IONs were more extensively and uniformly internalized by the breast cancer cells in suspension than the GNTs. Based on TEM images (Figure 4.3) and uptake data, the latter would certainly appear to be true.
Figure 4.6 Fluorescence microscopy images of magnetically sorted cell fractions. (a,d) Sorted fractions from a mixture of green ION-labeled and red unlabeled MC1 cells resulting in (a) first, eluate and (d) second, retentate fractions. (b,e) Sorted fractions from a mixture of green GNT-labeled and red unlabeled MC1 cells resulting in (b) eluate and (e) retentate fractions. MC1 cells were incubated on an external magnet. (c,f) Sorted fractions from a mixture of green ION-labeled and red unlabeled LM2 cells resulting in (c) eluate and (f) retentate fractions.

To further quantify the differences between GNT-labeled and ION-labeled cells, the cells eluted in each fraction were counted in order to calculate a percentage of cells eluted, nonmagnetic versus magnetic. As predicted by fluorescence microscopy, 90-100% of ION-labeled cells eluted in the second fraction, whereas only ~20% of GNT-labeled MC1 cells eluted in the second fraction. Considering the differences in composition of the two particles, these results are not surprising. With small (~5-10 nm
diameter) iron oxide cores, ION particles are superparamagnetic, with single magnetic domains and have higher magnetic susceptibility. This enables them to be efficiently retained in the high-gradient magnetic fields of the MACS® column. GNTs, however, are more complex mixtures of magnetically-active components. The paramagnetic Gd$^{3+}$ ions would only be weakly responsive to a magnetic field, as individual magnetic moments of each ion are randomly oriented. Any significant magnetic response would likely arise from the remnant catalyst particles of ferromagnetic (or superparamagnetic, for nanoscale particles of) nickel, which are partially removed from the US-tubes by HNO$_3$ purification prior to Gd$^{3+}$ loading. However, retention of GNT-labeled cells was greatly enhanced by placing an external permanent magnet beneath the culture plate during incubation. This could be due to an enhanced magnetization of the GNTs in the presence of an external magnetic force, possibly even aligning the magnetic moments within the particles to produce a larger net magnetic moment sufficient to improve retention on the MACS® column, however, further experimentation would be required to explain the enhanced retention without enhanced GNT uptake.

GNTs also exhibited an interesting effect as cell labels. For the labeling protocol, cells were plated in ultra-low attachment flasks or well plates, floating in cell culture medium supplemented with GNTs or IONs. In the case of the GNTs, labeled cells would start to adhere to the bottom of the flask, largely unable to be removed even after treatment with 0.25% trypsin-EDTA. The ultra-low attachment flasks are coated with a proprietary hydrogel that prevents cell attachment under normal conditions. GNTs, however, may be attracted to this hydrogel by nature of either their hydrophobic carbon cages or their hydrophilic Pluronic® surfactant coating, depending on the nature of the
hydrogel. Regardless of mechanism, this effect renders the cells “sticky,” but also largely unattainable post-labeling since GNTs are stuck to the bottom of the flask. However, since ICP and TEM both confirmed GNT uptake, the lack of magnetism cannot be blamed on this “stickiness.”

As can be seen in Figure 4.7, the presence of an external magnet during incubation has a significant effect on column retention. In fact, GNT-labeled cells without the presence of an external magnet were no more magnetized than unlabeled cells. One possible explanation of this was higher GNT uptake in cells loaded in the presence of an external magnet. In order to test this, Gd content was measured by ICP in cells labeled with and without an external magnet present during labeling (Figure 4.8). However, there was no significant difference in GNT uptake; thus, it cannot explain enhanced retention on the MACS® column. An alternative explanation could be that the individual magnet moments of the GNTs, or even perhaps the GNTs themselves, align in the presence of an induced field, thus creating a net magnetic moment large enough to be captured on the ferromagnetic column.
Figure 4.7 Magnetic retention properties of unlabeled and magnetically labeled SUM159 cells. * p < 0.001. No significant difference was observed between unlabeled and GNT-labeled cells, in terms of retention on MACS® column.
4.4.3. Magnetophoretic mobility of ION-labeled cells

Given the weaker magnetic properties and stickiness associated with GNT-labeled cells, ION particles were used exclusively for further development of the method to magnetically isolate quiescent breast cancer cells. In order to characterize the actual magnetic response of individual cells to an applied magnetic field (as opposed to just retention properties), CTV was used (Figure 4.9a and Figure 4.10). Results indicated a broad distribution of magnetophoretic mobilities in cells labeled in the same concentration of medium (Figure 4.10), even though cells labeled in concentrations as low as 1 µg Fe/mL (corresponding to ION uptake of ~2×10^5 IONs/cell) could be entirely captured on the magnetic column (Figure 4.9b). This indicates a lower specificity, but
high sensitivity of the MACS® column for isolating cells that are magnetized by the ION particles. The mobility dose-response curve revealed increasing mobilities up to a saturation point.

Figure 4.9 Dose-response curves of (a) magnetophoretic mobility and (b) percent retention on MACS® column of LM2 cells at various ION labeling concentrations.

Figure 4.10 Distribution of magnetophoretic mobilities of ION-labeled LM2 cells at concentrations of 0, 1, 5, 10, and 50 µg Fe/mL.
4.4.4. Capture of quiescent breast cancer cells

Once the ability to capture ION-loaded cells was established, the actual isolation of non-dividing cells could begin, as hypothesized in Figure 4.1. In initial studies, MC1 cells were labeled with 50 µg Fe/mL and plated at $10^5$ cells/mL in non-adherent culture for up to 30 days prior to magnetic separation. Even after 30 days, 100% of cells retained magnetism, suggesting that the IONs were either not losing label at a substantial rate due to slow division or that the initial labeling concentration was too high (Figure 4.11a). Since MC1 are xenograft tumor cells that have not been immortalized for long-term in vitro culture and their mitotic kinetics are not well known, SUM159 cells were used instead, and at a lower labeling concentration of 15 µg Fe/mL, to try to distinguish between slow division and high labeling concentration. Even after the initial labeling concentration was decreased, the cells still retained magnetism for at least 30 days (Figure 4.11b), indicating, at least, that the IONs were not being expelled from cells to any great extent.

One possible reason for the magnetic retention in ION-labeled cells over time is that they reached a critical confluence and stopped dividing. Throughout almost every experiment, cells were cultured in suspension in order to preserve any stem-like nature present in the cells, generating mammospheres instead of flat sheets of cells like those grown adherently. However, once the mammospheres reach a critical size, they become contact-inhibited and stop growing, as for confluent cells grown adherently. In order to generate cultures that lost enough magnetism to distinguish between dividing and non-dividing cells, LM2 cells were ION-labeled at low (10 µg Fe/mL) concentrations and
plated adherently (Figure 4.11c). In this case, the percentage of magnetic cells (retentate) declined significantly over several passages. In order to translate this effect for cells in suspension, initial cell densities were decreased from earlier studies using $10^5$ cells/mL to $2 \times 10^4$ cells/mL (Figure 4.11d). This alteration was sufficient to observe a decline in magnetism over time.

Although these figures do not represent a direct comparison from one panel to the next, the clear message is that as cells divide, they can lose enough IONs to no longer be retained on the MACS® column, enabling the capture of the relatively higher ION-loaded cells.
Figure 4.11 Magnetic retention properties over time. (a) MC1 cells, ION-labeled at 50 μg Fe/mL and incubated in nonadherent culture at an initial density of 3×10^5 cells/mL. (b) SUM159 cells, ION-labeled at 15 μg Fe/mL and incubated in nonadherent culture at 3×10^5 cells/mL. (c) LM2 cells, labeled at 10 μg Fe/mL and propagated adherently at a plating density of 4×10^4 cells/mL at each passage. (d) LM2 cells, labeled at 10 μg Fe/mL and incubated in nonadherent culture at 2×10^5 cells/mL.

In order to confirm that cells eluting in the magnetic fraction have enhanced magnetophoretic mobility over nonmagnetic cells, CTV was performed for MACS® fractions of LM2 cells that had been incubated for one month, with passaging every 10 days. At the collection point, 9% of cells comprised the magnetic fraction. As indicated in Figure 4.12, magnetic cells had low magnetophoretic mobilities. However, they were about twice as high as cells that eluted in the first, nonmagnetic fraction. This indicates
that the MACS® column can distinguish between magnetically distinct cells populations under these conditions.

![Bar chart showing magnetophoretic mobility of MACS fractions of LM2 cells propagated for one month in suspension. A significant enhancement in mobility was observed for the magnetic label-retaining fraction.](image)

Figure 4.12 Magnetophoretic mobility of MACS fractions of LM2 cells propagated for one month in suspension. A significant enhancement in mobility was observed for the magnetic label-retaining fraction.

Although magnetic retention is a strong indication of quiescence, it does not rule out the possibility of isolating cells that had begun with ION loads higher than other cells. In order to test whether the magnetic fraction is indeed quiescent, the two fractions were compared for uptake of EdU, which only incorporates into actively synthesizing DNA (Figure 4.13). As predicted, the magnetic fraction had significantly less EdU uptake than the nonmagnetic fraction, thus confirming the isolation of a relatively quiescent subpopulation of LM2 breast cancer cells.
Figure 4.13 Comparison of EdU uptake in MACS fractions by flow cytometry (LM2 cells) (a) DAPI-only cells, used to set EdU negative gate. (b) DAPI-labeled ION-high MDA231 control cells, used to set EdU low gate. (c) Percent of EdU positive cells in unsorted, (d) nonmagnetic (eluate), and (e) magnetic (retentate) fractions. (f) Percent EdU positive cells in each fraction. * p < 0.0001; ns = not significant.
4.5. Conclusions

Quiescence plays a crucial role in both normal and cancer cell biology. Despite the pre-existence of many different approaches, a facile, universal, and function-based method for isolating quiescent cells for further analysis has until now proven elusive. In this work, a new approach based on label retention of iron oxide nanoparticles to isolate a subpopulation of slow-cycling breast cancer cells was introduced. Depending on the surrounding environment, cells will either divide and lose the iron oxide label by dilution or remain dormant and retain the magnetism. Relying on readily available materials, this method combined the quiescent cell’s property of label retention with magnetic capture on a ferromagnetic column. Although untested, this method could theoretically be adapted to any cell type.

In addition to the establishment of a magnetic method to isolate quiescent cells, we have demonstrated that GNTs, while useful for MR imaging and retention of cells in tissue, may not possess the susceptibility sufficient to capture internally-labeled cells on a magnetic column fitted only with a permanent magnet with a field of approximately 0.6 T. However, by exposing cells to an external magnetic field during the labeling process, some magnetism could be induced such that a fraction (~20%) of the GNT-label cells were captured on the column.

4.6. Limitations and future work

Despite the advances presented, some limitations remain. First, a cell’s position in (or exclusion from) the cell cycle is acutely dependent on its microenvironment.
Quiescence is not, by definition, a permanent state of a cell, so isolating them in this manner only gives a snapshot of the current conditions being tested. In order to obtain a cell fraction with biological relevance, cells should be propagated *in vivo* to recapitulate the actual niche. Also, while easy to use and accessible, MACS® does not offer selectivity. As demonstrated, a wide range of intracellular ION concentrations facilitate entrapment on the magnetized column. A more sensitive system, such as those developed by Chalmers and Zborowski,\textsuperscript{124,125} could be employed or customized to isolate smaller ranges of label-retaining cells. Nevertheless, this work has opened new possibilities for exploring as yet undiscovered properties of an elusive cell population.
Chapter 5

Properties of magnetically-isolated quiescent breast cancer cells

5.1. Introduction

While the isolation of quiescent cells is an important contribution in and of itself, the motivation to obtain a population of slow-cycling cells stems from inquiry into the biological function of quiescent cancer cells, specifically. While conventional cancer therapies generally treat all tumor cells alike, clinical outcomes and research have demonstrated that, like normal tissue, most tumors are inherently heterogeneous. Moreover, some cancer cells are resistant to treatment and can cause recurrences long after the bulk of a tumor have been eradicated. In order to account for these observations, a stem-like model for cancer development, proposed over a century ago, has gained traction in recent years, leading to new approaches to study and potentially treat various types of cancer, from leukemia to breast cancer.
The cancer stem cell (CSC) model defines CSCs as cells with the ability to reproduce copies of themselves (self-renewal) and asymmetrically divide into daughter cells of reduced potency (differentiation). In experimental practice, CSCs are characterized as tumor cells that can regenerate new tumors, which consist of more than one cellular phenotype, when transplanted into animal hosts. Such tumor initiating cells (TICs) constitute a tiny portion (less than 1%) of the overall tumor population. Building evidence has indicated that quiescent cancer cells may be enriched for TICs or CSCs.

Moore and Lyle reviewed the accruing evidence for the property of quiescence in CSCs. Guo and coworkers demonstrated that a relatively less proliferative (in vitro) population of CD24+ ovarian tumor cells was able to differentiate into both CD24+ and CD24- cell types, expressed higher levels of stem cell genes, had enhanced chemoresistance, and formed tumors, as opposed to their CD24- counterparts. In another study, label-retaining pancreatic adenocarcinoma cells formed tumors at a rate ten times higher than non-label retaining cells. Pece et al. obtained a genetic profile for label-retaining normal mammary cells, then showed that breast cancer cells possessing this signature had enhanced tumorigenicity.

The focus of this work is the biology of breast cancer, in which the CSC model has demonstrated relevance. To date, there is no clear answer as to whether breast CSCs are relatively slow-cycling or, conversely, whether slow-cycling tumor cells have enhanced tumorigenicity. Furthermore, regardless of whether quiescent cancer cells exhibit increased tumorigenicity, reversible dormancy is a plausible explanation for drug
resistance exhibited in many cell types. Therefore, quiescent tumor cells represent a

crucial target for future cancer therapies. In this work, our aim was to further explore the

biological function of quiescent breast cancer cells, specifically of the LM2 breast cancer
cell line. LM2 cells were derived from MDA-MB-231 breast cancer cells that had

metastasized to the lungs in immunodeficient mice. \(^{134}\) This cell line was chosen for two

main reasons. First, MDA-MB-231 cells have low expression of claudin proteins, a major

component of tight junctions between cells. The claudin-low subtype has been
demonstrated as enriched for TICs. \(^{111,112}\) Secondly, research in the Lewis lab has shown

that LM2 and MDA-MB-231 cells have higher TIC frequencies than SUM159 cells
(approximately 1 in 6636 cells compared to 1 in 572 cells). \(^{130,135}\) Here, we tested drug

response, mammosphere-forming potential, and tumor initiation in immunodeficient

mice.

5.2. Methods

5.2.1. Docetaxel resistance study

LM2 breast cancer cells were ION-labeled overnight at 10 \(\mu\)g Fe/mL. Magnetic

retention on the MACS column was measured, and the cells were replated in suspension

at \(2 \times 10^4\) cells/mL in medium treated with 0.5 ng/mL, 5 ng/mL and 25 ng/mL docetaxel

and compared to untreated samples as well as samples with an equivalent volume of

DMSO as in the treated samples. Cells were allowed to propagate for 10 days as

mammospheres, then digested into single-cell suspensions via 0.05\% trypsin treatment.

Magnetic retention was then measured again.
5.2.2. Mammosphere formation assay

In order to evaluate mammosphere formation efficiencies (MSFE), a modified version of the established procedure\textsuperscript{136} was used. Breast cancer cells were centrifuged at 300 rcf, washed with PBS to remove protein content from the medium, and resuspended in 0.05% trypsin-EDTA for 5 min in a 37 °C water bath. In the case of evaluating MSFE over time, LM2 cells were passaged every 9 days, being plated at 2×10^4 cells/mL in MEGM+ with 0.5% methyl cellulose at each passage. Single cell suspensions were then plated in ultra-low attachment 6-well or 24-well plates at a density of 5×10^3 cells/mL in MEGM+ supplemented with 0.5% (w/v) methyl cellulose. Samples were incubated at 37 °C and fed 100 µL MEGM+ twice per week until they were evaluated 10 and 14 days after plating. Mammospheres were imaged and counted using a GelCount (Oxford Optronix) instrument. MSFE was calculated according to Equation 5.1.

\[
MSFE = \frac{\# \text{ MS per well}}{\# \text{ cells plated per well}} \times 100
\]

Equation 5.1 Calculation of mammosphere formation efficiency

5.2.3. Limiting-dilution cell transplantation study

ION-labeled LM2 cells were propagated for one month, MACS\textsuperscript{®} sorted, and serially diluted into PBS at twice the desired concentration for each dose and kept on ice prior to injections. Serially diluted doses of 5000, 1000, 500, 100, and 50 cells were injected into the number four mammary fat glands, which had been cleared of the endogenous mammary fat pad,\textsuperscript{137} of SCID/Beige female mice, in a 1:1 PBS:Matrigel v:v
ratio at 10 μL per injection. Results reported include data from a combination of two batches of 36 mice each.

5.3. Results & Discussion

5.3.1. Docetaxel enriches magnetic label-retaining population

Since magnetic-retaining cells are less proliferative than their nonmagnetic counterparts, the percentage of magnetic cells in a sample should be greatly enriched in samples treated with chemotherapeutic agent docetaxel. The mechanism of docetaxel in breast cancer cells is based on the stabilization of microtubules and inhibition of mitosis, leading to cell death. As a further demonstration of ION retention in quiescent cells, LM2 breast cancer cells were treated with 0.5 ng/mL, 5 ng/mL and 25 ng/mL docetaxel and compared to untreated samples as well as samples with an equivalent volume of DMSO as in the treated samples. As hypothesized, docetaxel killed the majority of the cells in the treated samples, but all remaining cells were magnetic (Figure 5.1). In untreated and DMSO treated samples, the cells divided as usual and only remained about 45% magnetic after 10 days of incubation. This finding further corroborates the effectiveness of the magnetic method for isolating quiescent cells in vitro.
Figure 5.1(a) Percentage of magnetic LM2 cells remaining 10 days after treatment for untreated cells, DMSO control-treated cells, 5 ng/mL docetaxel-treated cells, and 25 ng/mL docetaxel-treated cells. (b) Absolute cell counts of magnetic and nonmagnetic fractions after treatment.
Unfortunately, this study cannot definitively prove the intrinsic resistance of magnetically-isolated quiescent cells to docetaxel. The results shown can be the result of either prevention of cell division in treated samples or destruction of dividing cells leading to sole survival of ION-retaining cells. Future studies should first isolate ION-retaining cells after one month in incubation, then compare viability in response to various conventional therapies. However, the results here suggest that the isolated magnetic cells post-treatment can be used as a target for the development of new cancer therapy regimens.

5.3.2. Quiescent cells have no significant increase in mammosphere formation efficiencies

Sphere-forming assays have been utilized in normal and cancer biology as an *in vitro* method to enable stem-like cells to avoid differentiation that can occur in adherent culture and to predict stem-like content *in vivo*.\(^{136,139}\) At the outset of this work, the hypothesis was that quiescent cells would be enriched for stem-like breast cancer cells, which would thereby exhibit higher MSFE due to a greater ability to generate new cell colonies. Before testing this, the effect of magnetic label on MSFE was evaluated. SUM159 cells were labeled for 8 h with 50 μg Fe/mL or 30 μM Gd GNTs (on external magnet), washed, then incubated for 7 days prior to MS plating. As no significant difference was observed in MSFE (Figure 5.2), the magnetic label was excluded as a potential confounding factor in the MSFE of magnetically-isolated quiescent cells.
As ION-labeled LM2 cells were incubated and passaged *in vitro*, MSFE was evaluated at various time points. Even at the one-month mark, where 8% magnetic cells remained, no significant difference was observed in MSFE between unsorted, nonmagnetic (eluate), and magnetic (retentate) samples (Figure 5.3). Based on these results alone, the implication is that for LM2 cells propagated in non-adherent culture, the quiescent subpopulation is not enriched for stem-like content relative to more rapidly proliferating cells. However, sphere-forming assays have many drawbacks, namely that the *in vitro* environment may not possess the chemical factors present that can stimulate quiescent cells to reenter the cell cycle. Furthermore, MSFE does not always correlate well with TIC content, which must then be evaluated separately to corroborate or
oppose the *in vitro* sphere-forming assay. This can be seen by a comparison of MSFE and TIC frequency data for SUM159 and LM2 cells (Table 5.1).

![MSFE comparison between unsorted and magnetically-sorted fractions of LM2 cells after one month incubation as mammospheres post-ION-labeling (10 µg/mL). Unpaired t-test with a 95% confidence interval indicated no significant difference between MSFE for any fraction.](image)

Table 5.1 Comparison of TIC frequencies and MSFE for LM2 and SUM159 cells cultured *in vitro* and *in vivo*.130

<table>
<thead>
<tr>
<th>Cell preparation</th>
<th>LM2</th>
<th></th>
<th>SUM159</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% TIC</td>
<td>% MSFE</td>
<td>% TIC</td>
<td>% MSFE</td>
</tr>
<tr>
<td><em>in vitro</em></td>
<td>0.123</td>
<td>0.347±0.124</td>
<td>0.003</td>
<td>0.203±0.0512</td>
</tr>
<tr>
<td><em>in vivo</em></td>
<td>0.175</td>
<td>0.605±0.214</td>
<td>0.015</td>
<td>0.336±0.109</td>
</tr>
</tbody>
</table>
5.3.3. **Assessment of tumor formation in quiescent versus non-quiescent breast cancer cells**

Prior to the limiting dilution assay, a “mock sort” experiment was performed to estimate a range of cell doses for injections. Adherently grown LM2 cells were either ION-labeled or unlabeled, plunged through the MACS® column as a mock sort, and injected into SCID/Beige mammary glands, one on each side, with three mice per group. The results (Table 5.2) indicated that a range of 1000-10,000 cells per injection was reasonable. However, for the actual experiment, lower doses were added to see if the limits of the assay could be pushed.

Table 5.2 Limiting dilution transplant data for mock-sorted unlabeled and labeled LM2 cells, nine weeks post-injections in SCID/Beige mice.

<table>
<thead>
<tr>
<th>Mock sort</th>
<th># Tumors/# Transplantations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td># of LM2 cells</td>
<td>10,000</td>
</tr>
<tr>
<td>Unlabeled</td>
<td>3/4 (75%)</td>
</tr>
<tr>
<td>ION-labeled</td>
<td>3/6 (50%)</td>
</tr>
</tbody>
</table>

ION-labeled LM2 cells were propagated under nonadherent conditions for one month prior to injections to evaluate TIC content via the limiting dilution assay. Mice were monitored for tumor formation for 4-6 months post injections. One unanticipated complication was the widespread development of infections in many of the mice, for which 38% (excluding those that had formed tumors) had to be euthanized before the 4-month mark. The largest single contribute to mouse euthanasia was an infection that
manifests itself as a “head tilt,” where the head of an affected animal remains permanently tilted to one side. As shown in Figure 5.4, 60% of the mice were euthanized for reasons irrelevant to the study.

Figure 5.4 Cause of death in limiting dilution experiments, by percent of the 72 total mice transplanted. Other causes not specified included various infections such as pinna necrosis and signs of ill-health such as ruffled fur and hunched backs.

Despite this major loss of experimental data, limiting dilution results are reported in Table 5.3. Unfortunately, such low tumorigenicities for all samples precludes any definitive conclusions as to the absolute or relative TIC frequencies of any fraction. These low take rates in unsorted as well as sorted fractions could be attributed to several possible factors, including low cell doses, damage to cells through sorting (though the same cell populations that were used for injections were capable of both mammosphere formation and adherent growth), or long-term in vitro nonadherent culture conditions.
Future studies should start by employing higher cell doses, such as 5,000-50,000 cells per injection. Before this can be accomplished, however, the issue of the head tilt infection should be addressed. In addition, the cell model used for \textit{in vivo} studies can also be re-examined in light of the enrichment of TICs in STAT3-positive MDA-MB-231 and SUM159 cell lines.\textsuperscript{135} Results from that study suggest that the SUM159 cell line would have been a better model for the limiting dilution transplant studies shown here due to their higher STAT3 expression.

Table 5.3 Limiting dilution transplant data for sorted MACS fractions of LM2 cells, six months post-injections. Estimates for TIC frequency were calculated with the extreme limiting dilution analysis webtool, which also concluded no significant differences between the TIC frequencies of the fractions.\textsuperscript{140}

<table>
<thead>
<tr>
<th># of LM2 cells</th>
<th># Tumors/# Transplantations (%)</th>
<th>1/TIC frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,000</td>
<td>0/1 (0%)</td>
<td>7689</td>
</tr>
<tr>
<td>1,000</td>
<td>1/2 (50%)</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>0/2 (0%)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0/2 (0%)</td>
<td></td>
</tr>
<tr>
<td>Nonmagnetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,000</td>
<td>2/3 (67%)</td>
<td>2811</td>
</tr>
<tr>
<td>1,000</td>
<td>1/4 (25%)</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>1/4 (25%)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1/3 (33%)</td>
<td></td>
</tr>
<tr>
<td>Magnetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,000</td>
<td>1/4 (25%)</td>
<td>7237</td>
</tr>
<tr>
<td>1,000</td>
<td>0/3 (0%)</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>1/3 (33%)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1/3 (33%)</td>
<td></td>
</tr>
</tbody>
</table>

One separate limiting dilution study, however managed to evade serious losses due to premature mouse euthanasia. In this study, the same parameters were employed, with the exception of 20 days (instead of 30) incubation of ION-labeled LM2 cells prior to incubation. In this particular experiment and time point, 6.8% of the cells were magnetic at the time of injection, and an end point was instituted four months post-injections. Doses tested were 1000, 500, 100, and 50 cells, with three mice injected per group, two number 4 mammary glands (one on each side) per mouse. Tumor formation results are shown in Table 5.4. Again, the doses studied were likely too low to yield
useful information about the actual nature of the TIC frequencies among experimental groups.

Table 5.4 Limiting dilution transplant data for sorted MACS fractions of LM2 cells, four months post-injections. Estimates for TIC frequency were calculated with the extreme limiting dilution analysis webtool, which also concluded no significant differences between the TIC frequencies of the fractions. Due to the lack of tumor formation in the nonmagnetic group, no TIC frequency was reported.140

<table>
<thead>
<tr>
<th># of LM2 cells</th>
<th># Tumors/# Transplantations (%)</th>
<th>1/TIC frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000 500 100 50</td>
<td></td>
</tr>
<tr>
<td>Unsorted</td>
<td>0/6 (0%) 0/6 (0%) 0/6 (17%)</td>
<td>9875</td>
</tr>
<tr>
<td>Nonmagnetic</td>
<td>0/6 (0%) 0/6 (0%) 0/6 (0%)</td>
<td>n/a</td>
</tr>
<tr>
<td>Magnetic</td>
<td>1/6 (17%) 0/6 (0%) 1/6 (17%)</td>
<td>4666</td>
</tr>
</tbody>
</table>

Keeping the severe limitations of both TIC studies in mind, results were combined (calculating based on a four month end point for consistency). As shown in Figure 5.5, no significant difference was observed between unsorted, nonmagnetic, and magnetic cells in terms of TIC frequencies. By repeating this experiment at higher cell doses under conditions with reduced infection rates in mice, future studies can verify whether this preliminary outcome is the true result of quiescent LM2 cells having no outstanding tumorigenicity. The combined results of the present sphere-formation assay and limiting dilution assay suggest that those LM2 breast cancer cells that are quiescent in nonadherent culture conditions are not enriched for stem-like cell content.
Figure 5.5 Tumor take rate for MACS fractions of LM2 cells. Data is combined from Table 5.3 and Table 5.4. Error bars represent standard error of the mean. Unpaired t-tests with 95% confidence intervals indicated no significant differences between groups.

5.4. Conclusions & Broader Impact

This work exemplifies the kind of questions that can be answered through the isolation and functional analysis of quiescent cells. The drug-resistance in a quiescent, label-retaining fraction of LM2 breast cancer cells has been demonstrated. Further exploration was made into the stem-like nature of the quiescent subpopulation through sphere-forming and limiting dilution assays. While these preliminary results indicate a lack of stem-like enrichment in the quiescent, magnetic-label retaining group, the limitations discussed above necessitate further inquest. Despite advances, no conclusive
link has been established between nonadherent *in vitro* quiescence and stem-like cell content either *in vitro* or *in vivo*. The door for pertinent follow-up studies, however, has now been opened. Hopefully, future studies can build on this work and lead to impactful medical advances.

One immediate improvement that can be made to current work is testing the inherent drug resistance in quiescent cells. While we have already established that treatment of LM2 breast cancer cells *in vitro* with docetaxel enriches the sample for magnetic label retention, it remains unclear whether the long-term slow cycling cells are in fact more resistant than their proliferating counterparts. Follow-up studies should allow ION-labeled cells to proliferate as mammospheres for one month, separate the nonmagnetic and magnetic cells, and treat each fraction separately to directly compare drug resistance. To further improve the biological relevance of the study, cells could be propagated as tumors *in vivo*, digested, separated, and tested for resistance to various treatment regimens. Preliminary results for the tumor take rates of ION-labeled LM2 cells that were propagated *in vivo*, rather than as mammospheres *in vitro*, were 3 tumors out of 4 transplants and 2 tumors out of 4 transplants for unlabeled and ION-labeled LM2 cells, respectively.

A second extension to the present studies would be to enhance the comparison of tumorigenicity between proliferating and quiescent breast cancer cells. As mentioned above, cell doses should be increased to account for the low take rates that have been observed for LM2 cells cultured in nonadherent suspension for one month prior to injections. Alternatively, ION-labeled tumor cells should be cultured *in vivo* to allow for
true cell signaling to occur, rendering a much more accurate environment for the selection of quiescent cells. Since the microenvironment is crucial to the maintenance of stem cell behavior, the movement of all studies from culture flasks to animal models will be a necessary step toward realizing the full impact of this work.

Hand-in-hand with monitoring tumor formation, the tracking of metastases should also be addressed. Quiescent cells may have the ability to leave the initial tumor site and establish deadly tissue networks in other part of the body. Using the exact same experimental model shown here, the simple addition of full-animal fluorescence imaging could determine the spread of cancer throughout the mouse. LM2 cells express GFP, and could thus be tracked in a small animal model. Alternatively, fluorescently-tagged iron-oxide particles or other colored fluorescent proteins could be used to avoid overlap with green tissue auto-fluorescence. For large animals and humans, however, the minimal penetration depth of fluorescence would be insufficient. Fortunately, IONs themselves are strong $T_2$-weighted MRI contrast agents, and can thus be tracked non-invasively at full-body penetrating depths. The major drawback of using MRI is the low sensitivity, which would likely be incapable of identifying single-cell metastases. A radiolabel could be conjugated to the intracellular magnetic particle instead to enable highly sensitive positron-emission tomography (PET) to detect metastases instead. By testing the right combination of contrast agents and imaging modalities, the metastatic potential of quiescent cells should be able to be identified.

Another way to confirm stem-like content in quiescent cells is to compare with established biomarkers. While the ALDH positive, CD24$^{\text{low/neg}}$/CD44$^{\text{high}}$ phenotypes have
been demonstrated to enrich for TICs in breast cancer models, they are not universally expressed. However, STAT3 signaling may be a more promising marker to indicate CSC content, as measured in claudin-low breast cancer subtypes.\textsuperscript{135} By employing the lentiviral fluorescent STAT3 signaling reporter that was developed, transduced SUM159 cells can be ION-labeled and subsequently magnetically separated into quiescent and non-quiescent cells. Fractions can then be compared for STAT3 signaling enrichment as another indicator of stem-like properties. If quiescent cells are enriched for TIC content, they should also be enriched for STAT3 expression.

If quiescent cells prove to be enriched for drug resistance, tumorgenicity, or metastatic activity, they will be rendered prime targets for new therapeutic developments. Before they can be selectively destroyed, they must be fully understood. Quiescent and proliferating cells should be compared in terms of their genetic signatures, through quantitative polymerase chain reaction studies (qPCR) and RNA sequencing. The results could identify previously unknown biomarkers that can be selectively targeted in next-generation drugs. No matter whether they have any additional tumorgenicity, quiescent cells are dangerous because they inherently evade the very process of cell division that is the crux of most current therapies. One counter-intuitive method to be tested is to use a mitogen to stimulate quiescent cells back into the cell cycle, thus sensitizing them to current therapies. Another approach is to use magnetic or radio-frequency-induced hyperthermia. Chemotherapy-resistant cancer stem cells have been shown to be susceptible to infrared-induced hyperthermia. In the ION-labeled quiescent LM2 cells employed for this work, magnetically-induced hyperthermia may be the best treatment to
explore, due to the inherent superparamagnetism of the particles. Magnetic fields, unlike infrared radiation, are full-body penetrating, and would selectively target iron oxide nanoparticles. Moreover, extensive research has already been explored in the field of magnetically-induced cancer hyperthermia,\textsuperscript{141–143} specifically with iron oxide particles; whereas nanomaterial-mediated near-infrared\textsuperscript{144} and RF-induced methods\textsuperscript{145,146} are emerging technologies.\textsuperscript{147}

In terms of impact, there is no restriction of any of these studies to breast cancer alone. The magnetic method established for the isolation of quiescent cells should be easily extended to any cancer type, especially those with established immortalized cell lines. Functional assays can then be employed to ascertain stem-like cell content, thus establishing future therapeutic targets. Tumor dormancy, resistance, recurrence, and metastasis are all serious barriers to the eradication of cancer. Through the identification, isolation, and treatment of tumor cell subtypes that have inherent drug resistance and the stem-like abilities to self-renew and differentiate, these barriers can be drastically reduced. Quiescent cancer cells have proven elusive targets for isolation and therapy that can awaken to become silent killers. The present magnetic method for the isolation of quiescent breast cancer cells and subsequent functional studies may represent an important stepping stone toward the development of more successful cancer therapies in the future.
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