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Intracellular MRI Contrast Agents for High Magnetic Fields

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INTRACELLULAR MRI CONTRAST AGENTS FOR HIGH MAGNETIC FIELDS

By

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ABSTRACT

MRI magnets are constantly evolving with technologies for higher fields, improving sensitivity and increasing resolution. Major achievements in MRI magnet technology in recent years include the successful construction of a 21.1-T, 900-MHz magnet system built at the National High Magnetic Field Laboratory and the soon to be completed series connected hybrid system capable of reaching 36 T. However, contrast agents used in MRI today are mostly based on iron oxides and gadolinium, which both have limited high field properties. A full assessment of the high field properties of existing contrast agents as well as alternate paramagnetic options, such as dysprosium, is required to better utilize these compounds for biomedical applications.

This dissertation involves the evaluation of existing intracellular MRI contrast agents at high magnetic fields as well as the development of a novel bimodal contrast agent optimized for these high fields. The focus is on the performance of the agents with emphasis on cell labeling and tracking in biological systems.

This dissertation will provide background on contrast agents and their relaxation properties as well as the cell lines and animal models used. An assessment of commercially available iron oxide particles as intracellular contrast agents was performed utilizing a rat microglia cell line. Internalized iron particles were imaged in tissue mimicking phantom at two high magnetic fields and evaluated based on contrast generated with increasing iron dose or cell concentration. Results show that iron oxide has limited benefit at higher magnetic fields mainly due to saturation below 1 T. The labeling of human mesenchymal stem cells (hMSC) with the same super-paramagnetic iron oxide nanoparticles was performed to evaluate uptake, viability, proliferation and differentiation for in vivo implantation.

To improve upon commercial agents, a novel bimodal contrast agent based on dysprosium and quantum dots was fabricated and analyzed. These nanoparticles were developed using the quantum dot not only as a fluorescent agent, imparting bimodal imaging capabilities, but also as a platform for increasing the number of Dy$^{3+}$ that can be conjugated and delivered on a single nanoparticle to increase relaxivity. These particles have at least comparable $T_2$ contrast to existing iron oxide agents, and the potential for increased improvement with advent of fields above 21.1 T (Rosenberg et al. Magn. Reson. Med., 64 (3) 2010).
Bimodal fluorescent particles based on iron oxide were applied to in vivo animal models of traumatic brain injury for cellular tracking of an endogenous cell population, the neural progenitor cells of the subventricular zone, as well as exogenous, pre-labeled hMSCs. Data suggests increase labeling or migration of endogenous cell populations while exogenous labeled cells track to site of neuronal injury following an intra-arterial injection into the carotids. The impacts of these findings are discussed with respect to high field cell tracking in neurodegenerative processes.
CHAPTER ONE

INTRODUCTION

The very first step towards the development of Magnetic Resonance Imaging (MRI) were taken in 1973 when Nobel Laureate Paul Lauterbur discovered that a spatially varying magnetic field applied across a sample will alter Larmor frequencies, the precessional rates at which nuclear spins rotate in an applied magnetic field (1). During a relative short time period, MRI has developed to an important clinical tool with which patients now can be evaluated non-invasively with everything from heart and brain angiography to brain tumors to dynamic brain function by functional MRI, diffusion and fiber tracking. Unlike X-ray and other common imaging modalities, MRI does not use any ionizing radiation that can be harmful to tissues due to the molecular bond breaking power of the x-rays. Today, state-of-the-art magnet technology and increasing field strength create great opportunities to uncover in vivo events in the brain, down to a level where cellular activity can be monitored. These opportunities come with new challenges, especially with respect to MRI contrast agents that have to be used to track single cells.

This dissertation describes the development of a multimodal contrast agent optimized for high field MRI (11.75 – 21.1 T) as well as an evaluation of existing contrast agents with these novel high magnetic field systems. Potentially, the multimodal high field agent can be used as an intracellular tracking and monitoring device for in vitro and vivo applications. This novel agent incorporates chelated paramagnetic lanthanides and fluorescent nanomaterials (e.g. quantum dots) to visualize cell location and with the potential to monitor cell viability (e.g. intracellular pH, temperature). The application of this agent will provide an important technique to assess neurodegeneration in pre-clinical models.

Acute neurodegenerative injuries account for a significant proportion of neuronal damage in the patient population. In the US alone, 795,000 Americans suffer from ischemic stroke while another 1.4 million experience traumatic brain injury (TBI) on a yearly basis. These injuries have both an immediate impact on neurobiology and long term chronic degeneration that affect the overall health and well being of patients. Beyond the immediate loss of neuronal tissue is a more pervasive loss of at-risk tissue that can extend beyond the initial injury. While little can be done...
to regenerate or savage the initial sites of acute injury, it is often a crucial fight against time to save at-risk tissue in the penumbra. A real time, in vivo tracking and monitoring system of immune and tissue damage response to acute injury will aid in understanding the steps of disease progression and provide possible windows for therapeutic treatments.

1.1 Background

MRI is a non-invasive imaging method that can be used for a wide variety of applications from standard clinical diagnostic imaging to research applications such as flow measurement, diffusion and cellular imaging. Unlike X-ray and other common clinical imaging modalities, MRI foregoes ionizing radiation by utilizing nuclear spins that will align preferentially in the direction of an applied magnetic field and precess about the axis of that applied field. Once perturbed from alignment with the main magnetic field (B₀), there are two major relaxation mechanisms that return spins and the resultant bulk magnetization to thermal equilibrium: spin-lattice and spin-spin interactions, denoted by the relaxation time constants T₁ and T₂, respectively. These relaxation mechanisms can be used in MRI to develop image contrast because of the differential relaxation times developed in different microenvironments, such as tissues. With the appropriate timing of acquisitions during these relaxation processes, contrast can be developed between tissues with different apparent T₁ and T₂ values.

Prior to any acquisition, the thermal equilibrium state of the bulk magnetization vector of a spin system is parallel to the main field B₀, which typical is designated as the z direction. With the application of a radio frequency (RF) pulse, energy is put into the spin system so that spins are transferred to a higher energy state quantum mechanically and the bulk magnetization vector classically is tipped away from its original z alignment into the transverse xy plane. A longitudinal recovery of the bulk magnetization to the z direction will begin to occur after the RF pulse is released. The time constant denoted by T₁ is related to the recovery rate of the bulk magnetization to alignment with B₀. The energy imparted by the RF pulse is transferred to the environment or lattice from the spin system. Meanwhile, the T₂ time constant describes the rate of the loss of phase coherence within the spin ensemble due to interactions between the spins, which represents an entropic release of energy and decay of the bulk magnetization.
1.2 Contrast Agents

In MRI, image contrast and quality is dependent on a number of factors. Largely, the MR signal depends on the local physical composition of the sample and sequence parameters used during acquisition. These factors influence the dominant contrast mechanisms that are at work in a given image. Contrast can be further enhanced by introducing an exogenous compound, commonly referred to as a contrast agent. By employing the contrast agent, an increased signal difference between areas of interest and the background is created.

MRI contrast agents mainly affect the $T_1$ and $T_2$ values and contrast agents are often categorized according to these two groups (2). $T_1$ contrast agents generally lower the $T_1$ value, thus increasing the relative signal in a given area to generate positive contrast. On the other hand, $T_2$ contrast agents produce a negative contrast in which the $T_2$ value is shortened. Both $T_1$ and $T_2$ agents exploit paramagnetic metals to induce relaxation changes in the immediate vicinity of the compound. The most commonly used clinical agents are based on gadolinium (Gd) ions chelated with peptides to reduce the otherwise toxic effects of Gd$^{3+}$. Superparamagnetic iron oxide (SPIO) agents utilize paramagnetic iron to achieve primarily $T_2$ contrast. In all cases, the contrast achieved with a paramagnetic contrast agent depends on its relaxivity, which is illustrated by the following expression (3)

$$\frac{1}{T_{1,2,obs}} = \frac{1}{T_{1,2,d}} + r_1[CA]$$  \hspace{1cm} \text{Eq. 1}

$1/T_{1,2,obs}$ = the observed $T_1$ or $T_2$ value

$1/T_{1,2,d}$ = diamagnetic contribution of the $T_1$ or $T_2$

$r_1$ = relaxivity ($1/T_{1,2,p}$ = paramagnetic contribution)

$[CA]$ = concentration of the Contrast Agent

The relaxation rate is linearly dependent on the local concentration of the paramagnetic compound. A number of other factors also affect contrast such as temperature, water accessibility and pH.
1.2.1 Paramagnetic Contrast Agents

Paramagnetic materials magnetize only when an external magnetic field is applied. Materials that are classified as paramagnetic materials are those that have ions with unpaired electrons. Most common are lanthanide ions such as gadolinium (Gd\(^{3+}\)), dysprosium (Dy\(^{3+}\)) etc. Gadolinium is the most commonly used because of its seven unpaired electrons, the symmetric electronic state and high relaxivity. Gadolinium is today the most extensively used paramagnetic contrast agent and have properties very suitable to provide a good contrast because of electronic relaxation and high solubility (3, 4).

It is commonly known that Gd as a free ion is highly toxic and since the high doses needed for \textit{in vivo} contrast, will disturb the osmolality balance and lead to cellular damage. Therefore an extensive number of chelates have been synthesized. These chelates are made of ligands that make the Gd\(^{3+}\) stable and can radically reduce toxicity (2-4). For example, Gd-DTPA (gadolinium- diethylene- triamine- pentacetic acid) (5) which is a contrast agent that passively or non-specifically is introduced to the blood the blood stream. Gd-chelates have successfully been used to enhance contrast in order to image a specific cell. Two possible entrapment path ways for the chelates have been investigated: phagocytosis and pinocytosis, both which have been successful. Pinocytosis uses the cell process of absorbing particles through small vesicles, endosomes. Cells are incubated in media with Gd-chelates during a determined time period and will then absorb sufficient amount of Gd-Chelates in order to create enough MR contrast. Both Gd-HPDO3A and Eu-HPDO3A are examples of conjugations that have been proven to successfully be entrapped in the endosomal system without any effects on cellular functions (6). Internalization by phagocytosis is slightly different. Phagocytotic uptake can be induced by negatively charged molecules that are easily taken up by the cell or by labeling with receptor-mediated endocytosis, manly through avidin-biotin conjugation for cell specific uptake (7).

Despite the extensive use of Gd, it has a major downside; it has a decreased relaxivity at higher magnetic field strength as reported by Woods \textit{et al} (7). Woods \textit{et al} show that even at clinical field strength (1.5 – 3.0T) the effectiveness of Gd is limited and drastically decreased as the field strength increases (Figure 1).
As MRI magnets evolve and higher magnetic fields comes available especially with the newly commissioned 21.1 T (900 MHz), the highest field available for MR microscopy (8) at the NHMFL new contrast agents with higher efficiency must be engineered. Dy has been proven in contrary to Gd to improve relaxation in higher magnetic field strength as shown in Figure 3.

**Dy-DTPA-BBMA**

**Figure 1:** Illustration of R₁ relaxivity with two Gadolinium complexes. Woods M, *et al.* (7)

**Figure 2:** G.A.Walter, S.Santra, B.Thattaliyath and S.C.Grant, Nanoparticles in Biomedical Imaging: Emerging Technologies and Applications, J.W.M. Bulte and M.M.J Modo (Eds.), Springer (2008)
Dy, also a member of the lanthanoid group, has been used extensively in MRI as a chemical shift reagent (9). Recently, ionic Dy$^{3+}$ has received attention as a potential candidate for high field applications because the magnetism of Dy-complexes is impacted largely by Curie relaxation, which manifests as a large static magnetic moment at high magnetic field. To understand the impact of magnetic field on water relaxation in the presence of Dy$^{3+}$, it is necessary to evaluate the effects of paramagnetic relaxation as well as exchange. Relaxation is affected on both the outer sphere (i.e. water molecules diffusing in close proximity to the Dy$^{3+}$ ion) and the inner sphere (i.e. relaxation induced by the temporary binding of water molecules). Previous studies have already in depth described in the mechanisms involved in the efficacy of Dy as a high field contrast agent (10-12) and the spin relaxation theory for Dy has been laid out by Freed J.H. 1977 (13) and Gillis P. et al 1999 (14). A detailed explanation will therefore not be given here. In short, the increase in mainly T$_2$ contrast but also T$_1$ is due to the Curie relaxation effect that originates from the dipolar interaction of water molecules and the static magnetic moment from electrons (15). For water, the apparent longitudinal (R$_1$) and transverse (R$_2$) proton relaxation rates increase with field strength due to the contribution of Curie relaxation, which has shown a squared dependence with the external magnetic field. For Dy-complexes, R$_2$ relaxometry has displayed an increased T$_2$ contrast with magnetic fields compared to Gd (16, 17). Likewise, a squared dependence with field has been demonstrated for R$_1$ relaxation in both Dy$^{3+}$ ions and Dy-complexes for fields up to 18.8 T (10, 18). The main group of actors in the proton longitudinal and transverse relaxation for Dy at high fields are the correlation times modulating the dipolar interaction ($\tau_{c1,2}$).

The R$_{1p}$ proton relaxation rate can be expressed as the summation of inner (IS) and outer sphere (OS) relaxation contributions according to:

$$R_{1p} = R_{1p}^{is} + R_{1p}^{os}.$$  \hspace{1cm} Eq. 2

The IS relaxation depends on the interaction between Dy and directly bound water molecules. It can be expressed with the molar concentrations of Dy and water, the mean residence lifetime of the coordinated water molecule exchanging with the bulk water pool ($\tau_m$), the molar metal complex (q) and the bound longitudinal relaxation ($T_{1m}$) according to:
\[
R_{1p}^{i} = \left[\frac{[Dy]q}{[H_{2}O]}\right]\left(\frac{1}{T_{1m} + \tau_{m}}\right).
\]

Eq. 3

\(T_{1m}\) can be expressed as \(\frac{1}{T_{1m}} = \frac{1}{T_{1m}^{DD}} + \frac{1}{T_{1m}^{C}}\), where the dipolar interaction (\(T_{1m}^{DD}\)) and Curie dipolar contribution (\(T_{1m}^{C}\)) can be expressed, respectively, as:

\[
\frac{1}{T_{1m}^{DD}} = \frac{2}{15} \left(\frac{\mu_{0}}{4\pi}\right)^{2} \gamma_{i}^{2} \mu_{B}^{2} g_{j}^{2} S(S+1) r^{-6} \left(\frac{3\tau_{c1}}{1 + \omega_{l}^{2}\tau_{c1}} + \frac{7\tau_{c2}}{1 + \omega_{s}^{2}\tau_{c2}}\right)
\]

Eq. 4

\[
\frac{1}{T_{1m}^{C}} = \frac{2}{5} \left(\frac{\mu_{0}}{4\pi}\right)^{2} \gamma_{i}^{2} B_{0}^{4} \mu_{B}^{4} g_{j}^{4} S(S+1) r^{-6} \left(\frac{1}{(3k_{B}T)^{2}}\right) \left(\frac{3\tau_{cc}}{1 + \omega_{l}^{2}\tau_{cc}^{2}}\right)
\]

Eq. 5

In Eqs 4 and 5, \(\mu_{0}\) is the permeability of free space, \(\mu_{B}\) is the Bohr magneton, \(\gamma_{i}\) is the proton gyromagnetic ratio, \(B_{0}\) is the magnet field strength in Tesla and \(g_{j}\) is the Lande factor of Dy equal to 1.333 (10). The total spin quantum number is described by \(S\), \(T\) is the absolute temperature, \(k_{B}\) is the Boltzmann constant, \(r\) is the geometric factor that describes the average distance between the metal and coordinated water molecule (observed nucleus), and \(\omega_{l}\) and \(\omega_{s}\) are the angular procession frequencies for the proton and electron, respectively. The correlation times modulating the dipolar interaction (\(\tau_{c1,2}\)) are the inverse sum of the rotational relaxation time (\(\tau_{r}\)), electronic relaxation times (\(\tau_{s1,2}\)) and \(\tau_{m}\):

\[
\frac{1}{\tau_{ci}} = \left(\frac{1}{\tau_{r}} + \frac{1}{\tau_{m}}\right) + \frac{1}{\tau_{Si}} = \frac{1}{\tau_{cc}} + \frac{1}{\tau_{Si}}.
\]

Eq. 6

For Dy, \(\tau_{s1,2}\) are constant, equal and field independent; thus, \(\tau_{s} = \tau_{s1} = \tau_{s2}\), which is on the order of \(10^{-13}\) s. \(\tau_{m}\) and \(\tau_{r}\) are longer, making \(\tau_{c}\) primarily dependent on \(\tau_{s}\). As shown in Eq 6 the correlation time for the Curie contribution (\(\tau_{cc}\)) is dependent only on \(\tau_{r}\) and \(\tau_{m}\), with \(\tau_{r}\) dominant for small Dy complexes (10).
In the same fashion, the OS contribution for Dy is the sum of the dipolar \(1/T_{1\text{OS}}^{DD}\) and the Curie contribution \(1/T_{1\text{OS}}^{C}\) were the respective contribution is described as (10):

\[
\frac{1}{T_{1\text{OS}}^{DD}} = \frac{C_{DD}}{aD} \times \left\{ \frac{S(S + 1) - S_c \coth \frac{X}{2J} - S_c^2}{2J} \cdot (j_0(\omega_1, \tau_d, \tau_r) + 7 \cdot \coth \frac{X}{2J} \cdot S_c \cdot j_0(\omega_1, \tau_d, \tau_r)) \right\} \quad \text{Eq. 7}
\]

\[
\frac{1}{T_{1\text{OS}}^{C}} = \frac{32\pi}{45000} \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\gamma_i^2 \mu_B^2 g_j^2 N_A [Dy]}{aD} S_c^2 \left( j^2 \omega_1 \tau_D \right) \quad \text{Eq. 8}
\]

where \(C_{DD}\) is equal to:

\[
C_{DD} = \frac{16\pi}{135000} \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\gamma_i^2 \mu_B^2 g_j^2 N_A [Dy]}{aD} \quad \text{Eq. 9}
\]

\(N_a\) is Avogadro’s number, \(a\) is the distance of closest approach between the Dy molecule center and water molecules, and \(D\) is the sum of water and paramagnetic complex diffusion coefficients. \(J(\omega)\) is the spectral density function described in detail by Aime \textit{et al} (16). \(S_c\) is the time averaged Curie spin (14), \(\chi\) is the spectral density function for dipolar interaction (10, 13).

At low magnetic field strength the OS term is primarily that of diffusion time constant \(\tau_s\) while at high fields the Curie effect modulated with the translational correlation \(\tau_d (\tau_d = a^2/D)\) will become more significant. \(\tau_d\) together with \(\tau_r\) are the two components that is important for longitudinal relaxation at high field expressed through the inner- and outer sphere relaxations as described by Elst \textit{L. et al} (10, 11).

The proton transverse relaxation \(R_2^{2p}\) is also dependent on the OS \(R_2^{\text{OS}}\) and IS \(R_2^{\text{IS}}\) relaxation. The IS is described in a similar manner as Eq. 3 when it is assumed that the water exchange is slow \(\tau_m^{-1} \leq \Delta \omega_M\) and \(\tau_m^{-1} \gg T_2^{-1}\) which occur at high fields and \(\tau_M\) becomes field independent (10) and consequently the IS relaxation is not dependent on the dipolar and Curie contributions:

\[
R_2^{2p} = \frac{[Dy]}{[H_2O]} q \frac{\tau_m \Delta \omega_M^2}{\tau_m \Delta \omega_M^2} \quad \text{Eq. 10}
\]
Here, $\Delta \omega_M^2$ is the chemical shift of the coordinated water molecule. The dipolar and curie OS relaxation are described as:

$$R_{2OS}^{DD} = \frac{C_{DD}}{aD} \cdot \left\{ \left[ S(S+1) - S_c \coth \frac{\gamma}{2J} - S_c^2 \right] \cdot (3 \cdot j_0(\omega_s, \tau_d, \tau_s) + 4 \cdot j_0(0, \tau_d, \tau_s)) \right\}, \quad \text{Eq. 11}$$

$$+ \frac{C_{DD}}{aD} \cdot 6.5 \coth \frac{\gamma}{2J} \cdot S_c \cdot j_0(\omega_s, \tau_d, \tau_s)$$

$$R_{2OS}^{C} = \frac{16\pi}{45000} \left( \frac{\mu_0}{4\pi} \right)^2 \gamma^2 \mu_b^2 g^2 N_a [C] \times \frac{1}{aD} \cdot S_c^2 \left\{ 3 \cdot j^4(\omega_l, \tau_D) + 4 \cdot j^4(\omega_0, \tau_D) \right\}, \quad \text{Eq. 12}$$

The Curie effect has been reported with Dy-DTPA chelates which illustrate that the magnetic field and residence time of coordinate water molecules effects the proton transverse relaxation rates at high magnetic fields and will depend on $B_0^2$ and $\tau_m$ until $(\Delta \omega_M^2)^{-1}$ get close to $\tau_m$ (10).

Dy$^{3+}$ is classified is predominantly a negative contrast based on the high field effect on water protons. Dy$^{3+}$ aqua ions have however shown to have enhanced longitudinal ($T_1$) relaxation at higher field strengths due to the Curie contribution as described above were they express the inverse total longitudinal relaxation as the sum of the inverse longitudinal dipole and curie contribution according to Eq 5.

$T_1$ contrast is more desirable in brain imaging mainly due to the relative long $T_1$ of brain tissue and short $T_2$. An increase in $R_1$ would create a signal enhancement where the Dy-agent is present in contrary to a $T_2$ agent were the signal void can easily be confused with other susceptibility artifacts. Based on the relaxivity properties of Dy$^{3+}$ it should be possible to achieve $T_1$ as well as $T_2$ contrast with a Dy-based contrast agent.

### 1.2.2 Superparamagnetic Contrast Agent

The majority of intracellular contrast agents consist of the Superparamagnetic agents which are normally iron oxide nanoparticles These agents act as superparamagnetic materials that affect MR relaxation (principally $T_2$ and $T_2*$), that results in a signal loss that corresponds to the location of the labeled cell. These particles behave similar as to paramagnetic particles but they can do so even above the Curie temperature, which is the temperature where paramagnetic
materials normally lose its magnetic properties. Furthermore, iron oxide agents are commercially available, and are readily endocytosed by numerous cell lines. However, the negative contrast imparted by these agents reduces the overall information content of the MR image by eliminating critical signals relevant to cellular viability and performance. Iron oxide particles range from the very smallest, with a diameter less than 50nm (Ultra-small superparamagnetic iron oxide, USPIO) to the larger with a diameter greater than 200nm. SPIOs’ generally have a diameter around 50nm.

Super paramagnetic iron oxides (SPIOs), which are composed of small (1-10-nm scale) crystallite regions (19), provide domains that are thermodynamically independent. However, in a magnetic field ($B_0$) and contrary to the single atom alignment of paramagnetism, these domains align with the $B_0$ field as a single unit. This whole-domain alignment generates microscopic field gradients that dephase neighboring protons due to increased magnetic susceptibility (20). The dephasing of spins creates not only a hypointense signal at the site of the SPIO but also causes a hypointense signal in nearby tissue (21).

In cellular imaging, SPIOs demonstrated effectiveness even after coating the particle with macromolecular material such as dextran (22) (23), liposomes (24) and lectin (25) as well as chitosan, starch and polystyrene (2). As such, these materials can be used to target particular cell types, such as macrophages, that naturally phagocytose foreign objects in vivo. This technique works excellently when labeling leukocytes, lymphocytes and monocytes for immune tracking (26) or macrophages that are associated with immune diseases such as atherosclerotic plaque formation (27). SPIOs also have been modified to non-specifically target non-phagocytotic cells by conjugation with a transfection agent such as poly-L-lysine, protamine sulfate (28) or the HIV-Tat peptide (29). Bulte et al. (30) showed that it was possible to track stem cells in vivo with a non-specific targeting, magnetodendrimer with high affinity for the cell membrane. Specific targeting can be performed by modifying the SPIOs with cell-specific receptors, such as antibodies (21, 31-33).

The major benefit of SPIOs is the increased MR sensitivity resulting from susceptibility induced dephasing of proximal spins and the resultant negative contrast that is achieved. As a result, SPIOs function as either $T_2$ or $T_2^*$ agents. However, the magnetic susceptibility perturbation induced by SPIOs can be so pronounced, particularly at high fields that signal from surrounding soft tissue can be lost, causing a “blooming” of hypointensity around the
nanoparticle. This artifact effectively can reduce the information content of the MR scan by either destroying signal or masking other anatomical features (21).

1.3 In vitro applications

Contrast agents can be used readily to label cells in culture. Normally, such labeled cells then are implanted in vivo. However, the methods of transfection and intracellular localization of contrast agents within the cell are critical to the functionality (both as an MR-visible tag and as a therapeutic intervention) and viability of implanted cells. Several cell lines will be utilized in this described work. Each cell line has its own specific property with respect to the evaluation of cellular migration, propagation and functionality in in vivo animal disease models.

Neuronal Tera Carcinoma cells (NT-2), for example, have the ability to differentiate into CNS neurons but also the migratory ability associated with neurogenesis, which is of great interest when it comes to visualizing and tracking migratory pathways as well as brain plasticity. This cell line already has been tested successfully for nanoparticle uptake with Lipofectamine. In contrast, murine microglia (Bv2) and human mesenchymal stem cells (hMSC) do not require an external transfection agent to uptake nanoparticle staining. Bv2 cells are microglia with phagocytotic properties and are associated with many neurological diseases, which make this cell type useful in tracking cell migration related to immune response. hMSCs are a primary stem cell line that posses the ability to differentiate into chondrocytes, osteoblasts and adipocytes. They also have been shown to secrete chemokines in association with neuronal injury thus have potential for use in stem cell regenerative therapies. All of these cell lines have properties that can be utilized for implantation in animal models, for which their fate and transport can be tracked in real time and in vivo.

1.3.1 Bv2 Murine microglial cells

The choice of using a microglia cell line has many benefits when trying to study intracellular contrast agents. Microglia cells are effectively brain macrophages and play an important protective role in the central nervous system (CNS). In the parenchyma, microglia cells remove harmful pathogens and play an important role in inflammatory and injury in both acute and chronic neurodegeneration.
Microglia cells are known to take on different forms, associated with age and status of neurons in the brain parenchyma. Early in human development, microglia cells have an amoeboid shape with properties typical of a macrophage phenotype. It has been shown in rats that amoeboid microglia appear in a late stage of fetus development and disappear soon after birth (34). The amoeboid microglia is believed to migrate from mesodermal glial cells to the brain parenchyma during the embryonic development and from bone marrow-derived blood monocytes throughout the postnatal period (35). The amoeboid microglia have an important role in cleaning cell debris in the developing brain but also to “clean up” and remove neurons during normal remodeling of the fetal brain (36). Amoeboid microglia cells are precursors to the ramified (resting) microglia cell.

Ramified microglia are the most abundant type of microglia in the CNS and composes of about 10-20% of the total glial cell population. Ramified microglia cells have direct contact to neurons, blood vessels and astrocytes indicating that they play an important role in the support of neurons through cleaning of metabolites and toxic waste from dying or damaged neuronal cell bodies (37). It also has been suggested that ramified microglia cells may present multipotent stem cell characteristics that can give rise to neurons, astrocytes and oligodendrocytes (38).

Microglia are activated from the ramified stage when the CNS experiences an injury or is invaded by viruses, bacteria, fungi and parasites (35, 39). In the event of an injury, active microglia cells accumulate at the site of injury and phagocytose damaged cells and debris (40). They also excrete many anti-inflammatory mediators that help to coordinate the CNS immune response. Released anti-inflammatory factors include superoxide, nitric oxide, matrix metalloproteinase and tumor necrosis factor (35). There is a controversy regarding the neuroprotective versus neurodegenerative role of activated microglia. Even though there is no doubt that microglia play a crucial role in the fight against foreign organisms and protection of at-risk neurons, many neurological diseases such as Alzheimer’s disease, multiple sclerosis, Parkinson’s disease and Amyotrophic Lateral Sclerosis (ALS) are linked to chronic activation of microglia, causing a compromise in neuronal function and potentially increasing the severity of the disease.

The microphage nature of microglia cells as well as the close association with many neurological diseases makes this cell type very beneficial when it comes to uptake of intracellular contrast agents and tracking cell migration in vivo. Even the non-activated stage,
ramified microglia recently have been discovered to be very motile (37), which is promising for *in vivo* MRI cell tracking in early stages of neurodegenerative disease and possibly even neuro-regeneration by means of intracellular labeling. With *in vitro* experiments, microglia cells can be activated by lipopolysaccharides (LPS), a component of the outer membrane layer of gram-negative bacteria and a powerful stimulus of secretory (cytokines and chemokines) products in microglia cells (35).

### 1.3.2 Human Mesenchymal stem cells

Human mesenchymal stem cells (hMSC) are a multipotent stem cell line found in mesenchymal tissue such as bone, cartilage, muscle, ligament, tendon and adipose. They are derived from bone marrow and can be cultured *in vitro*. Their differentiation into tissue specific cells, including osteocytes, chondrocytes, stromal cells and adipocytes, is carried out by exposing adult hMSCs to different induction media and culture conditions. Due their ability to differentiate into the tissue generating cell lines, hMSCs can be used to restructure these distinct mesenchymal phenotypes (41). For example, autologous MSC in a scaffold have been transplanted into a bone repair site and regenerated bone tissue (42). In a similar manner, cells in hyaluronan, and polymeric scaffolds are use to produce cartilage tissue (43). These cells also have the potential to differentiate into neuronal and glia cells once exposed to epidermal growth factor (EGF) (44, 45). Due to their tissue regenerative properties, hMSCs posses the potential for *in vivo* tracking and migration followed transplantation. However, in order to visualize hMSC *in vivo* with MRI, they too need to be labeled with a contrast agent. SPIOs have been coated with cell penetrating peptides (CPP), like poly-L-Lysine (PLL), to facilitate uptake of the iron nanoparticle in MSCs. The PLL-iron oxide nanoparticle was able to visualize hMSC in gelatin sponges once implanted *in vivo* (46). The usage of a transfection agent or CPP, however, has been shown to be unnecessary for the uptake of SPIOs. Hsiao *et al.* showed that hMSCs were detectable in a 1.5-T scanner without any effect on viability, proliferation or differentiation. They determined that the intracellular iron content was 23.5 pg/cell when exposed to 100 µg/ml iron for 24 h (47). Interestingly, it has been reported that intravenously injected hMSC have localized to the site of ischemic brain tissue (48) as well as in infarcted heart 2-3 days following tissue damage (49). In addition, MSCs secrete many stimulatory factors for regulating inflammation.
processes, as well as neuronal growth factors in cell cultures of damaged brain tissue extracts (48). Recent theories suggest that the primary function of hMSCs is not to provide replacements units of tissue but rather to create a beneficial microenvironment for the regeneration of tissue (50). Regardless, these properties of hMSCs are useful in tracking the regeneration of neurons in association with traumatic brain injury or stroke.

1.4 In vivo applications

Tracking disease stages and neurological lesions can be valuable not only in finding a diagnosis of a disease but in reducing symptoms and improving treatments. Cell therapies have the potential to reduce disease symptoms and also the potential to regenerate damaged tissue. For traumatic brain injury (TBI), and other brain injuries like stroke have different causes, but their pathophysiology has many common denominators. Early detection and treatment of brain lesions can improve patient outcome drastically. Stem cells and microglial cells play an important role in the initial response to acute brain injury, and the ability of tracking early cell migration and activity is important for the understanding of the factors involved in palliative treatments, tissue regeneration and recovery. This section will give a summary of TBI and MRI methods commonly used to detect such an injury.

1.4.2 Traumatic Brain Injury

In many ways, TBI has similar pathological symptoms as ischemic stroke. In animal models, a TBI is generated by a weight falling with a pre-determined speed or concussion focused on the skull to induce a controlled cortical impact (CCI). TBI is a two stage injury, with the primary insult caused by mechanical impact forces that cause distortions and destruction. Especially damaging is the linear, spatiotemporal impact but other less damaging forces, such as rotational forces, are present. The gray matter is more impacted by the linear forces while deeper white matter is more affected by the rotational forces, which is also linked to concussion response (51, 52). The secondary injury is the biomolecular and physiological response. This response is what ultimately decides the extensiveness of the impact. One of the major causes of tissue damage in TBI is glutamate. The excitotoxicity from glutamate release is a consequence from alternated calcium homeostasis and oxygen damage. Glutamate is a neurotransmitter that is
released followed by Ca\(^+\) influx as regulated by NMDA (N-methyl-D-aspartic acid) and AMPA (-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (52, 53). However, it is unclear if glutamate is a product or cause of initial massive depolarization of neurons. The high intracellular influx of Ca\(^+\) has a devastating impact on cells and many organelles, such as mitochondria, that affect the formation of crucial ATP (54). Cytoskeleton and protease function also are disturbed after TBI (52). The secondary injury is linked to inflammatory responses that are mediated by neutrophils, activated microglia, macrophages, astrocytes and neurons (55). Csuka et al. showed that microglia cells are present as early as 4 hr post CCI (56). The inflammatory response is intended to have a beneficial role, but also can be harmful through the release of neurotoxins that induce additional brain damage. Macrophages (microglia) are known to play a important role in tissue damage repair (55, 57), and endogenous labeled macrophages with SPIOs have been used in CCI mouse models to track their migration and accumulation to the site of brain injury providing an in vivo 3D assessment of macrophage response (55). A better understanding of cellular response in secondary injury will lead to better treatment to save salvageable brain tissue.

1.5.4 MRI of TBI

MRI is a good tool to evaluate ischemic brain lesion mainly because of the formation of edema. The tissue damage can be imaged with diffusion- and perfusion weighted MR sequences (DWI and PWI) and also with regular T\(_2\)- and T\(_1\)-weighted images. These types of images evaluate the degree of ischemic lesion and assess treatments made to reduce or the effect of stroke. PWI provides information on blood flow deficits while DWI can estimate the degree of tissue damage.

Diffusion-encoded MRI is sensitive to the Brownian motion of the molecules under investigation, particularly in the presence of restrictions and hindrances introduced by tissue microstructure (e.g. cell membranes, macromolecules, proteins and fibers). The degree of restriction or hindrance is dependent on the tissue (e.g. gray versus white matter) and pathological modifications due to edema, tumors, etc. Diffusion-weighted sequences aim to capture the motion of water by adding a dephasing and rephasing gradient pair separated by some time increment that permits faster diffusing molecules to leave the frame of reference before they can be refocused. Therefore, in less restricted tissue, water molecules will be
dephased by the first gradient but not rephase by the second gradient, resulting in a significantly reduced signal magnitude. On the other hand, when water molecules are restricted, they will not be able to diffuse out of the imaging voxel and will experience both the dephasing and rephasing, resulting in much lower signal reductions. As described earlier, one of the main pathological response to both TBI and stroke is the formation of edema due to the rapid break down of the Na\(^+\)/K\(^+\) pump, consequently shifting water from the extracellular space to the more restricted intracellular space (58). When imaging this phenomenon with DWI, the increased water content in the cells will be more restricted, displaying an increased contrast particular to the damaged area and a decreased apparent diffusion coefficient (ADC) that reflects the restricted motion of water. In many ways the TBI injury resembles an ischemic lesion by the formation of the penumbra which visualized as a hyper intense area which after a couple of days retracts and forms an unsalvageable necrotic core. In pre-clinical animal models, ischemic lesions usually are evaluated as a function of time from initial insult, and the DWI is usually acquired in conjunction with an anatomical T\(_2\)-W image for comparisons between injured and uninjured subjects.

Omori et al have evaluated the effects of single vs. multiple doses of intravenously injected hMSC in MCAO animal models (59). They were able to evaluate the regression of volume lesions by diffusion- and T\(_2\)-weighted images. Figure 3 shows an example of DWI images illustrating the ischemic lesion by its hyperintensity shown in the right hemisphere.

![Figure 3: DW images from Omori et al (59) 6 hr post-MCAO surgery. Animals were scanned with DWI, yielding bright contrast corresponding to edema in the lesion. From left to right, the images display control (no injected hMSC) and then increasing doses of hMSCs.](image)

Another example, described by Toyama et al, used injections of gene modified hMSCs to evaluate structural recovery in MCAO. In this study, bone marrow derived hMSCs were
transfected with angiopoietin-1 (ANG) to evaluate the effect of angiogenesis, size of damage brain volume and stroke recover. The study concluded that either hMSCs alone, hMSC with ANG or hMSC transfected with a combination of the ANG and vasculature endothelial growth factor (VEGF) has beneficial therapeutic effects resulting in a reduced infarction volume, increased angiogenesis, and improvement in functional outcome (60).

Toyama et al also utilized PWI in their evaluation. With PWI, it is possible to evaluate the capillary microcirculation by measuring cerebral blood flow and volume. This technique utilizes dynamic pulse sequences, primarily $T_2/T_2^*$-weighted echo planar images (EPI), to measure blood perfusion by means susceptibility induced signal effects resulting from either exogenous contrast agents or by arterial spin labeling (API). In API, blood is saturated upstream of the selected slice, altering the magnetization of blood entering the imaging volume.

Together, diffusion and perfusion imaging provide measureable techniques for evaluating pathological tissue damage and recovery potential in CNS injuries related to TBI. As such, these techniques provide information about the pathophysiologival effects of acute neural degeneration as well as the salvageable penumbra and its treatability in stroke patients (61). Furthermore, with little modification, these same techniques can be used to track labeled cells (either implanted or endogenous) that may speed recovery and improve outcomes.
CHAPTER TWO

INTRACELLULAR SUPER PARAMAGNETIC IRON OXIDE AT HIGH MAGNETIC FIELDS

Iron oxide is one of the most commonly used MRI contrast agent. Superparamagnetic iron oxide (SPIO) coated with dextran, which is clinically known as Feridex, is the most used type and is usually applied for liver imaging. This iron particle can be readily taken up by cells without any external transfection agents; however, iron has limited benefit at higher fields due to the saturation at 1 T but might be beneficial at higher fields due to susceptibility effects. This chapter investigates the potential use of iron as intracellular contrast agent at high magnetic fields, namely 21.1-T the highest field strength available for MRI imaging.

Two types of cells are evaluated with SPIOs in this chapter, namely Bv2 and hMSC. Bv2 cells are focused mainly on contrast differences between field strengths and viability while hSMC is focused on the long term retention of SPIOs and viability. Respectively, these cell lines represent prototypical, microglia (Bv2) and adult stem cells (hMSC), with the later cell type demonstrating the potential to differentiate into other cell types.

2.1 Introduction

With the commissioning of stronger magnets, high field MRI use has increased in both clinical application (62-65) and animal research (66-68). New magnets with strengths above 14.1 T, culminating currently at 21.1 T (900 MHz) for MRI applications (8), create not only new opportunities for biomedical research but also many challenges, particularly with respect to optimized exogenous contrast agents. Superparamagnetic iron oxides (SPIOs) have well known contrast mechanisms for MRI, though their behavior and suitability for high field applications up to 21.1 T has yet to be evaluated. This study seeks to explore the high field contrast provided by intracellular SPIOs used to label in vitro mammalian neural microglia as a means of providing an early biomarker for acute neurodegeneration. To assess these agents, two high magnetic field strengths were employed, 11.75 and 21.1 T. These fields cover the range of pre-clinical, high
field magnets but also provide insight into the potential application of such techniques for high field human scanners.

The Bv2 microglia cell line employed are brain macrophages that play an important protective role in the central nervous system (CNS) by facilitating uptake of foreign particles through phagocytosis. In the parenchyma, brain microglia removes harmful pathogens and promotes tissue regeneration at a site of injury or inflammation. Microglia cells have direct contact with neurons, blood vessels and astrocytes, supporting neurons by clearing byproducts and toxic waste from dying or damaged cell bodies (37). Microglia cells recently have demonstrated extremely motility (37), which is promising for in vivo MRI cell tracking of early stage neurodegenerative disease. It also has been suggested that microglia cells may present multipotent stem cell characteristics with the possibility of differentiating into other cell types, namely neurons, astrocytes and oligodendrocytes (38). Although intended to provide a neural protective function, chronically activated microglia are associated with the progression of many neurological diseases such as Alzheimer’s Disease (AD), Multiple Sclerosis (MS), Parkinson’s Disease (PD) and Amyotrophic Lateral Sclerosis (ALS) (69), and this chronic activation compromises neuronal functional, potentially speeding the evolution of pathological deficits. As such, these cells are a prime candidate as an early biomarker of neurodegeneration, while also providing therapeutic potential and long term diagnostic value.

As exogenous contrast agents, SPIOs impact MR relaxation (principally $T_2$ and $T_2^*$), resulting in a phase-induced signal loss proximal to either individual nanoparticles or aggregates. Besides gadolinium chelates, SPIOs have received the most attention among contrast agents with respect to both clinical and pre-clinical applications (21, 26, 70-74) because of their high sensitivity and well known chemistry, particularly with respect to the facilitation of intracellular uptake and targeting specific cell types (22-26, 75, 76). SPIOs induce hypointense contrast by means of thermodynamically independent domains that align as a single unit in the presence of an external $B_0$ field. This whole-domain alignment generates microscopic field gradients that dephase neighboring protons due to increased magnetic susceptibility (20). The dephasing of spins decreases or eliminates signal not only at the site of the SPIO but also in surrounding tissue (21). However, the magnetic susceptibility perturbation induced by SPIOs can be so pronounced, particularly at high fields that signal from surrounding soft tissue can be lost irrevocably, causing a “blooming” of hypointensity around the nanoparticle.
The purpose of this chapter is to investigate the ability for a microglia cell line to phagocytose SPIO nanoparticles and compare the contrast generated by the intracellular localization at two high magnetic fields in a tissue mimicking phantom. These evaluations were performed with a commercially available SPIO and were conducted to assess the impact of iron loading and cell count on MR contrast as well as cell viability. In addition, hMSCs are evaluated with similar methods to investigate in the long term retention of SPIOs once internalized as well as the effect on viability and proliferation.

2.2 Material and Methods Bv2 cells

2.2.1 Cell culture

Bv2 cells were maintained in a 5% CO₂, 37º C incubator and grown in 75-cm² tissue culture flasks with Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen Corp, Carlsbad CA) supplemented with 10% non-essential amino acids, 10% heat inactivated Fetal Bovine Serum (Atlanta Biological, Atlanta, Georgia), 1% antibiotics/antimyotics (Gibco,Invitrogen Corp, Carlsbad, CA) and 0.1% gentamycin (Invitrogen Corp, Carlsbad CA). For each experiment, six-well plates (10-cm²/well) were seeded with approximately 50,000 cells. Cells were allowed to grow to 90% confluence before any treatment began. Prior to adding SPIO (Feridex, Bayer Healthcare, Wayne, NJ; 11.2 mg/mL of Fe) to the culture medium, cells were exposed to 10 µg/mL of lipopolysacchride (LPS, Sigma Aldrich, Inc., St Louis, MO) for 24 hr to activate the microglia. Then cells were incubated for 6 h with SPIOs before harvest. Three washes were performed with tris-buffered saline (TBS) before trypsination with TrypLE (Invitrogen Corp, Carlsbad, Ca) to ensure that no contrast agents were attached to the cell surface.

2.2.2 Sample preparation

MRI measurements were carried out by suspending the cells in a tissue mimicking phantom made with agarose gel. Agarose cell layers were formed by mixing an equal volume of cell suspension in media with a 2% (w/w) low-temperature agarose (VWR, Suwannee, GA) to form a 1% (w/w) agarose-cell final concentration. The suspended cells were layered in a 10-mm
NMR tube (Wilmad Glas Lab, Buena, NJ) with a 1% agarose layer separating the cell containing layers as shown in Figure 4.

Two types of experiment were performed to investigate intracellular contrast by SPIOs at 11.75 and 21.1 T. In the first experiment (SPIO dosing), Bv2 cells were incubated with different volumes of Feridex (1, 2 and 5 µL) corresponding to initial exposure doses of 11.2, 22.4 and 56 µg Fe. For each dose, 100,000 cells were immobilized in separate agarose layers. The second experiment (Cell count) was performed by incubating cells with the same volume of Feridex (5 µL) but generating individual agarose layers containing 25,000, 50,000, 100,000 and 200,000 cells.
cells. Each experiment was repeated (n = 3 or 4) for the each of the two magnetic field strengths. Cells were counted, and viability was measured on an Innovatis Cedex HiRes Cell Analyzer (Malvern, PA), which utilizes trypan blue dye exclusion to measure cell membrane integrity.

### 2.2.3 MR Experiments

MR images were acquired at 11.75 T (500 MHz) and 21.1 T (900 MHz). The 500-MHz magnet is an Oxford magnet with a widebore (WB) diameter of 89 mm while the 900-MHz system is an ultra-widebore (UWB) magnet with a diameter of 105 mm built entirely at the National High Magnetic Field Laboratory (NHMFL). Both magnets are equipped with Bruker Avance consoles and Micro2.5 gradients (Bruker Corp, Billerica, MA). Separate 10-mm Bruker birdcage coils, tuned to 500 and 900 MHz respectively, were used for imaging all samples.

Measurements were performed to quantify $R_1$ and $R_2$ relaxation for each sample. Common acquisition parameters for all sequences included: matrix = 128x128, BW = 75 kHz, FOV = 1.8x1.0 cm, slice thickness = 1.0 mm. For $R_1$ and $R_2$ measurements, a single slice 2D spin-echo (SE) sequence was used with varying TE (15-300 ms) and TR (25-15000 ms) times for the respective contrast weighting. High resolution $T_2^*$-weighted 3D gradient recalled echo (GRE) images also were acquired for each sample. For all experiments, the acquisition temperature was maintained at 23° C.

Magnitude images were analyzed using Regions of Interest (ROIs) drawn to cover each cell layer as well as the agarose spacing layers. The average ROI signal intensities were fitted by non-linear regression using the Levenburg-Marquadt algorithm in SigmaPlot 7.101 (SPSS Inc, Chicago, IL). For $R_2$ values, a three-parameter exponential decay function was employed while a three-parameter exponential growth was used for $R_1$. All regressions are fitted with a noise baseline to account for the rectified noise of magnitude images. 3D GRE images were used to measure signal intensity in each respective cell layer. These values then were normalized to agarose in accordance with Eq. 1 to measure the signal intensity relative to agarose in percent.

$$\% \text{ relative signal} = 100 \times \left(1 - \frac{S_A - S_B}{S_A} \right), \quad \text{Eq. 13}$$
where \( S_A \) is the signal from agarose and \( S_B \) is the signal from an individual cell layer.

### 2.2.4 Intracellular Iron Measurements

Concentrations of intracellular iron were determined with a high-resolution inductively coupled plasma mass spectrometer (ICP-MS) (FinniganMat ELEMENT I, Waltham, MA). Cell samples were dissolved in 50 \( \mu \)L of concentrated nitric acid and 50 \( \mu \)L of concentrated perchloric acid, dried to 150 °C and re-dissolved two times, once in 0.5 mL of 7N HNO\(_3\) followed by drying to 120 °C and once in 1 mL of 2% HNO\(_3\) followed ultrasonication, centrifugation and dilution by a factor of 10. Samples were run at medium resolution to separate the \(^{40}\text{Ar}^{16}\text{O}\) from \(^{56}\text{Fe}\). Both \(^{56}\text{Fe}\) and \(^{57}\text{Fe}\) were measured, and the isotope ratio was used to guard against interferences. Iron solutions (Alfa Aesar Specpure, Ward Hill, MA) of 1, 5 and 10 ppb were used as standards and drift monitors. Acquisition sequences were bracketed by blank and standard measurements after every 12 samples, and all data were blank corrected.

Prussian blue staining (Sigma Aldrich, St Louis, IL) was performed to visualize intracellular iron. Additionally, a SPIO particle (Bangs Laboratories Inc. Fisher, IN) coated with a fluorescent green molecule was used in conjunction with a red florescent protein targeted to the endosomes in order to investigate the intracellular location of phagocytosed particles.

### 2.2.5 Statistics

Statistical analysis was performed with SPSS 17.0 (SPSS, Inc. Chicago, IL). ANOVA statistical analysis was used to evaluate relaxation data for each respective magnetic field strength, and Tukey’s post hoc test was used to determine significance between each individual SPIO dose or cell count. Student’s T-tests were performed to assess the significance of each sample group between 11.75 and 21.1 T. Data was determined to be statistically significant at \( p < 0.05 \).

### 2.3 Materials and Methods hMSCs

Some of methods used for evaluating hMSCs are the same as described in previously such as sample preparation and MRI methods. Any additional methods used with the hSMC are covered in this section.
2.3.1 Cell culture

Standard frozen human bone marrow-derived stem cells were obtained from the Tulane Center for Gene Therapy and were cultured following a method outlined in our prior publications (77). Briefly, bone marrow aspirates from healthy donors ranging in age from 19 to 49 years were collected under an Institutional Review Board approved protocol. Plastic adherent nucleated cells were separated from the aspirate, expanded on TCP petri dishes using α-MEM supplemented with 10% FBS at 37 °C and 5% CO₂, and cultured to passage five. All cells used in the experiments in this paper were seeded at passage six.

Media samples were collected before each media change and stored at -50 °C until used for analysis. Glucose and lactate concentrations were determined using a 2500 Biochemistry Select Analyzer (YSI Incorporated, Yellow Springs, OH).

2.3.2 SPIO incubation

hMSCs, expanded as described above, were seeded at 4,000 cells/cm² on plasma treated six-well plates (BD Falcon, Franklin Lakes, New Jersey) in 3 mL of culture media. Twenty-four hours after plating, the cells were washed with sterile PBS, and then 3 mL of fresh media was added and then Feridex brand SPIO (Bayer Healthcare, Wayne, NJ; 11.2 mg/mL of Fe) was administered to each well at one of three different loadings: 0, 11.2, 22.4 and 56.0 µg of iron corresponding to 0, 3.73, 7.47 or 18.6 µg/mL, respectively. After 6 h of incubation the SPIO/cell culture medium was removed, the cells were washed 3 times with sterile PBS, and 3 mL of fresh culture medium was added to each well. Cells were then cultured for an additional 1, 7, 14 or 21 days with media changes every 3 days.

2.3.3 MTT

Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma Aldrich, Il) was used to assess cell viability. MTT was dissolved at 5 mg/mL in RPMI Media 1640 (Gibco, Carlsbad, CA), 0.8 µm filtered, and then stored at -20 °C until needed. Media was removed from samples, cells were washed with sterile PBS followed by the addition of phenol-free MEM with 10% FBS at the
same volume as the original culture volume. MTT solution was added to each well at 10% of the media volume, and the samples were then incubated at 37 °C and 5 % CO₂ for 3 hours. After incubation, the MTT containing media was removed and replaced with an equal volume of 0.1 N HCl in 100 % isopropanol under agitation. Once the formazan was completely dissolved, the supernatant was read on a microplate reader at 590 nm and quantified against a standard containing a known number of cells. All reported values are an average from three individual wells.

2.3.4 Prussian blue

In accordance with the manufacturer’s instructions, Prussian blue staining (Sigma Aldrich, St Louis, IL) was performed to visualize intracellular iron. Briefly, samples were fixed in 2.5% glutaraldehyde for 1 hour, washed with PBS followed by DI-H₂O, incubated with working solution for 10 minutes, followed again by a DI-H₂O rinse, placed in working pararosaniline solution, dehydrated in graded ethanol washings, and visualized on an Olympus IX70 (Center Valley, PA) with an Optronics (Goleta, California) camera attachment. The percent positive cells was calculated by taking a ratio of the total number of cells showing positive blue stain to the total number of cells from five fields of view with no less than 30 cells per field of view.

2.4 Results with Bv2 cells

2.4.1 SPIO uptake and cell viability

Prussian blue images display intracellular uptake of SPIOs by the Bv2 cells (Figure 5a). Figure 5b shows the fluorescent SPIO particle (green) and its co-localization within endosomes (red). Furthermore, intracellular uptake was verified with ICP-MS, for which an average iron loading per cell was measured. Data are presented in Table 1 after baseline adjustments.

No effect on viability was witnessed for Bv2 cells exposed to any concentration of SPIOs. In fact, viability measurements were found to be consistently above 95%, indicating that nearly all cells were alive when immobilized in 1% agarose.
2.4.2 SPIO dosing

SPIO exposure during incubation was intended to determine (a) if Bv2 cells could be labeled with different intracellular SPIO concentrations without exogenous transfection agents and (b) if differing intracellular SPIO concentrations provided enhanced contrast at 21.1 T over 11.75 T. As measured by ICP-MS (Table 1), the available SPIO concentration during exposure induced a strictly linear increase in intracellular iron content (adjusted $R^2 = 0.9991$).

**Figure 5:** a) Prussian blue staining of Bv2 cells. Blue staining indicates the presence of iron. b) Bv2 cells incubated with a micron-sized fluorescent SPIO (green) and co-registered for the endosomes (red).

**Table 1:** Summary of SPIO concentration and Bv2 uptake as measured by ICP-MS

<table>
<thead>
<tr>
<th>SPIO Dose (µg Fe)</th>
<th>SPIO concentration in media (µg Fe/mL)</th>
<th>Mass of Fe (pg/cell) after 6-h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.00</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>11.2</td>
<td>3.73</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>22.4</td>
<td>7.40</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>56.0</td>
<td>18.60</td>
<td>0.83 ± 0.04</td>
</tr>
</tbody>
</table>

Data are presented with the mean +/- standard deviation
Every dose (mass Fe/cell) showed significance against all other doses as determined by one-way ANOVA and Tukey HSD (p < 0.05)
For relaxation measurements (Table 2 and Figure 6), $R_1$ was insensitive to increased SPIO exposure, while $R_2$ increased slightly with intracellular iron concentration displaying $r_2$ relaxivities of 9.29 and 3.73 s$^{-1}$/pg/cell for 21.1 and 11.75 T, respectively. Compared to agarose, only the two highest doses at 21.1 T demonstrate significance in $R_2$ while all three doses show significance at 11.75 T. Between field strengths, only the 56.0-µg doses show significant differences in $R_2$. The longitudinal relaxations show no difference or significance between iron loading and field strengths. Figure 4 displays 3D GRE images acquired at 21.1 T of immobilized Bv2 cells incubated with the different iron doses to provide a qualitative representation of the contrast enhancement due to increased intracellular SPIO loading.

![Graph](image)

**Figure 6:** Graph illustrates relaxation rates vs. iron per cells for $R_1$ (○) ($r_1 = 0.71$ s$^{-1}$/pg/cell)) and $R_2$ (●) ($r_2 = 3.73$ s$^{-1}$/pg/cell)) at 11.75 T and $R_1$ (△) ($r_1 = 0.78$ s$^{-1}$/pg/cell)) and $R_2$ (▼) ($r_2 = 9.29$ s$^{-1}$/pg/cell)) at 21.1 T. $R_1$ is multiplied by a factor of 100 in order to show the two relaxation rates in the same graph.
2.4.3 Cell count

Figure 7 and Table 3 show the impact of increased cell concentrations on relaxation. Figure 4d displays a GRE image of a representative sample from the highest field strength, illustrating increased contrast with increasing cell number. Contrary to agent dosing, the $R_1$ relaxivity is lower (higher $T_1$) at 21.1 T compared to 11.75 T. For both fields, longitudinal relaxation as a function of cell count does not show any change with the number of cells, displaying a slope of $-7.16 \times 10^{-8} \text{s}^{-1}/\text{pg/cell}$ for 11.75 T and $-4.05 \times 10^{-8} \text{s}^{-1}/\text{pg/cell}$ for 21.1 T; the only significant difference in $R_1$ between fields was found for the 25,000 cell layer. Likewise, with respect to $R_2$, there was no statistical significance between fields except for the lowest cell count (25,000 cells), indicating that both the longitudinal and transverse relaxation were largely independent of field. The $R_2$ relaxations demonstrate similar slopes at both fields: $6.46 \times 10^{-5} \text{s}^{-1}/\text{pg/cell}$ and $7.45 \times 10^{-5} \text{s}^{-1}/\text{pg/cell}$ for 11.75 and 21.1 T, respectively. As shown in Table 3, the $R_2$ relaxation as a function of cell count demonstrates significant differences between counts and agarose at 11.75 T, but not at 21.1 T. Regardless, the trends and relaxivities at both fields were nearly identical, displaying increases with cell count.

Table 2: Relaxation rates from Bv2 cells layered with increasing initial mass of Fe during incubation

<table>
<thead>
<tr>
<th>Mass Fe [µg]</th>
<th>11.75 T</th>
<th>21.1 T</th>
<th>11.75 T</th>
<th>21.1 T</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30.06 ± 0.49</td>
<td>31.06 ± 1.08</td>
<td>8.90 ± 2.33$^a$</td>
<td>12.21 ± 3.80$^a$</td>
</tr>
<tr>
<td>11.2</td>
<td>32.38 ± 2.0</td>
<td>31.5 ± 0.48</td>
<td>12.27 ± 2.87$^c$</td>
<td>14.93 ± 3.20$^a$</td>
</tr>
<tr>
<td>22.4</td>
<td>32.27 ± 1.93</td>
<td>32.78 ± 0.69</td>
<td>12.20 ± 1.86$^c$</td>
<td>17.27 ± 2.32$^c$</td>
</tr>
<tr>
<td>56</td>
<td>32.64 ± 1.77</td>
<td>32.37 ± 1.28</td>
<td>14.74 ± 1.07$^{c,T}$</td>
<td>21.77 ± 3.71$^{c,T}$</td>
</tr>
<tr>
<td>Agarose</td>
<td>31.65 ± 1.74</td>
<td>32.8 ± 0.80</td>
<td>6.79 ± 1.04</td>
<td>9.17 ± 0.60$^{a,b}$</td>
</tr>
</tbody>
</table>

Relaxation data are presented with the mean +/- standard deviation.

$^a$Significantly different from 56-µg Fe incubation as assessed by Tukey HSD (p < 0.05)
$^b$Significantly different from 22.4-µg Fe incubation as assessed by Tukey HSD (p < 0.05)
$^c$Significantly different from agarose as assessed by Tukey HSD (p < 0.05)
$^T$Student’s T-test showing significance for individual Fe incubations between 11.75 and 21.1 T (p < 0.05)
Figure 7: Graph illustrates $R_1$ and $R_2$ relaxation rates versus cell count for $R_1$ (○) (slope $b_1 = -7.16 \times 10^{-8} \text{ s}^{-1} \text{cells}^{-1}$) and $R_2$ (●) (slope $b_2 = 6.46 \times 10^{-5} \text{ s}^{-1} \text{cells}^{-1}$) at 11.75 T and $R_1$ (▼) (slope $b_1 = -4.05 \times 10^{-8} \text{ s}^{-1} \text{cells}^{-1}$) and $R_2$ (▼) (slope $b_2 = 7.45 \times 10^{-5} \text{ s}^{-1} \text{cells}^{-1}$) at 21.1 T. $R_1$ is multiplied by a factor of 100 in order to show the two relaxation rates in the same graph.
2.4.4 Relative $T_2^*$ contrast

The relative susceptibility induced contrast measured with a 3D GRE acquisition shows obvious benefit for the higher field strength when comparing different intracellular SPIO labeling, while different cell counts show no increased detectability at the higher field. As demonstrated in Figure 8, the SPIO doses show significant differences between field strengths. At 11.75 T, the only significant contrast increase is seen between the highest and lowest loadings; at 21.1 T, no significant differences are evident between SPIO doses. For cell counts (Figure 8b), there is no significant difference between the two fields, and only the 50,000 and 200,000 cell layers at 21.1 T show significance contrast differences as a function of cell count.

Table 3: Relaxation times from Bv2 cells incubated with 56 µg of Fe and layered with increasing number of cells

<table>
<thead>
<tr>
<th>Cell count</th>
<th>Cell density / mL</th>
<th>$R_1$ [s⁻¹]</th>
<th>$R_2$ [s⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11.75 T</td>
<td>21.1 T</td>
<td>11.75 T</td>
</tr>
<tr>
<td>25000</td>
<td>208,333</td>
<td>46.06 ± 7.96$^T$</td>
<td>32.4 ± 1.7$^T$</td>
</tr>
<tr>
<td>50000</td>
<td>416,667</td>
<td>46.77 ± 8.65</td>
<td>34.2 ± 1.5</td>
</tr>
<tr>
<td>100000</td>
<td>833,333</td>
<td>46.12 ± 7.73</td>
<td>34.0 ± 1.4</td>
</tr>
<tr>
<td>200000</td>
<td>1,666,667</td>
<td>45.14 ± 8.13</td>
<td>32.3 ± 1.9</td>
</tr>
<tr>
<td>Agarose</td>
<td>46.06 ± 7.78</td>
<td>33.8 ± 2.0</td>
<td>7.02 ± 1.23$^{a,b}$</td>
</tr>
</tbody>
</table>

Relaxation data are presented with the mean +/- standard deviation.

$^a$ Significantly different from 200,000 cell layer as assessed by Tukey HSD (p < 0.05)

$^b$ Significantly different from 100,000 cell layer as assessed by Tukey HSD (p < 0.05)

$^c$ Significantly different from agarose as assessed by Tukey HSD (p < 0.05)

$^T$ Student’s T test showing significance for individual cell dosing between 11.75 and 21.1 T (p < 0.05)
Figure 8: Percent decrease in $T_2^*$ contrast versus iron content per cell where * demarks the significance with respect to the highest iron loading at 11.75 T. No significance is seen between individual iron loadings at 21.1 T. The symbol + indicates significance between field strengths for a given iron loading. 

b) Percent decrease in $T_2^*$ contrast versus cell count where the only significance shown is between 50,000 and 200,000 cell count for 21.1 T as indicated by *. No significance is seen between field strengths. All data are considered significant at $p < 0.05$. 
2.4 Results: hMSC

2.4.1 MRI

Figure 9 illustrates the contrast generated from the intracellular iron with hMSCs suspended in agarose layers during the 14-day time period. In Figure 9e,f, both $R_2$ and $R_2^*$ show a reduction of relaxation times ($s^{-1}$) over the entire time period. For $R_2$ at day one, the largest iron dose is significant different to the two lowest iron doses. In addition the 22.4-µg iron dose is significant to control (0 µg). For $R_2^*$, on the other hand, the 56-µg iron dose is significant to all other doses at day one. In addition $R_2^*$ show a significant decrease in relaxation at day 7 between the highest and lowest dose while 56-µg iron loading has decreased significantly over the 14-day time period. The reduction in $R_2^*$ relaxation time is confirmed with the $T_2^*$ weighted images shown in Figure 10 b-d. The reduction in transverse, $R_2$ contrast over the time period is presumably due to the dilution process of the iron particles during the two divisions that will occur during a 14-day time period. The reduced intracellular iron reduces the aggregation of iron particles per cell hence decreases susceptibility artifacts that are utilized for increased $T_2$ and $T_2^*$ contrast. It should be considered that $R_2^*$ is notoriously difficult to quantify where coil sensitivity, background perturbations and field inhomogeneities are evident impacting the significance calculations. Regardless, both relaxation measurements show trends of reduced contrast over the time period again, showing the dilution of iron particles. The dilution factor may in fact be stronger for the 56-µg loading for both $R_2$ and $R_2^*$.

As determined by MTT assay no affect on viability was seen during the time period and the different iron doses compared to control as seen in Figure 10.
Figure 9: Illustration of the cell-agarose layering in an NMR tube. b-d: 3D GRE images of immobilized hMSC at day 1 (b), 7 (c) and 14 (d). Figures e) and f) show relaxation graphs of $R_2$ (e) and $R_2^*$ (f) relaxation of each iron exposure over the 14-day time period. Brackets represent significant difference within each sample while dashed brackets indicate significance over the entire time period as measured by ANOVA and Tukey’s post hoc test (p<0.05).
Figure 10: Cell growth of SPIO labeled and non-labeled hMSCs. No statistical significance was found between iron exposures at each individual time point. However, there is significant cell growth for all iron loadings at day 14 compared to day 1 while the 22-µg loading at day 1 is significantly different to days 5 and 7 (p<0.05).

2.4.3 Prussian blue

Prussian blue staining showed iron incorporation after 6 hours of SPIO incubation for every concentration except the 0-µg sample, which did not show any positive signal for iron at any time point. Increasing SPIO concentration increased the percentage of cells having incorporated Feridex at all time points as seen in Figure 10 and Table 4. By day 14, all samples show a decrease in the percent positive cells, maintaining the pattern of increasing concentration having higher percentage positive cells.
Figure 11: Light microscope images of Prussian Blue stained hMSCs at day 1 (a-d), day 7 (e-h) and day 14 (i-l) after initial SPIO exposure. Left column represents control (0-µg Fe) (a,e,i), 11.2-µg Fe (b,f,j), 22.4-µg Fe (c,g,k) and 56.0-µg Fe (d,h,j) with all images at 10x magnification. The percent positive cells decreases over time for all samples except the control, which consistently tested negative for the presence of iron inside the cells (a,e,i).
Table 4: Percent of Positive hMSC from Prussian Blue Staining at Days 1, 7 and 14

<table>
<thead>
<tr>
<th>Initial Iron Exposure (mg)</th>
<th>Day 1 (%)</th>
<th>Day 7 (%)</th>
<th>Day 14 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11.2</td>
<td>$73.72 \pm 11.07^{a,b}$</td>
<td>$75.28 \pm 16.01^b$</td>
<td>$49.21 \pm 5.22^a$</td>
</tr>
<tr>
<td>22.4</td>
<td>$77.99 \pm 8.62^a$</td>
<td>$78.37 \pm 12.74$</td>
<td>$60.79 \pm 8.50$</td>
</tr>
<tr>
<td>56.0</td>
<td>$100 \pm 0^b$</td>
<td>$93.92 \pm 6.86^b$</td>
<td>$66.35 \pm 12.26$</td>
</tr>
</tbody>
</table>

All iron exposures are significantly different from controls (0-µg initial exposure)

*a* Significantly different from 56.0-µg initial exposure (p<0.05) for same time point

*b* Significantly different from day 14 (p<0.05) for same initial exposure

2.5 Discussion

2.5.1 Bv2 cells

This study investigates the cellular uptake of SPIO nanoparticles and explores the detectability of these labeled cells with regard to intracellular iron content and cell count in a tissue mimicking agarose phantom at two high magnetic fields. The purpose is not only to investigate the uptake potential and characteristics of Bv2 cells with respect to commercial nanoparticles but also to evaluate the information content of SPIO contrast at these field strengths.

The results suggest that SPIOs can readily be taken up by Bv2 cells without external transfection agents, solely through simulation with LPS. Further, the SPIO labeling had no impact on cell viability, which was measured to be higher than 95% for all iron loadings. The pathway of SPIO transfection is similar for all macrophage-like cell lines, namely through phagocytosis that sequesters nanoparticles in vesicles that ultimately localize in the perinuclear region, as seen in Figure 5. Macrophage uptake is highly dependent on the initial iron concentration at exposure (78), which also was reflected by the ICP measurement shown in
Table 1. Although the vesicular sequestration can negatively impact T$_1$ relaxation, as seen with intracellular T$_1$ quenching of paramagnetic lanthanides (72, 79, 80), the intracellular aggregation of SPIOs also increases susceptibility-induced perturbations that decrease T$_2$ and T$_2^*$ relaxation values (81).

Klug et al. showed that macrophages incubated for 4 h with rhodamine-covered iron nanoparticles at a concentration of 18.9 µg Fe/mL, similar to what is reported in this study, resulted in a 0.38 pg Fe/cell and R$_2$ relaxation of approximately 10 s$^{-1}$ at 17.6 T (82). Bulte et al. reported that magnetodendrimer incubated for 48 h at a 25-µg/mL iron concentration with HeLa cells resulted in a R$_2$ relaxation of 130 s$^{-1}$ (30) at 1 T while labeled MION-46L resulted in a R$_2$ relaxation rate of about 20 s$^{-1}$ at 1.5 T when incubated in CG-6 cells for 48 h with 50 µg Fe/mL and immobilized at a cell density of 2 x 10$^7$ cells/mL (20). The relaxation times reported above are larger than measured in this report but it should be noted that the iron concentrations and incubation time are higher than in this study.

The relatively short incubation time of six hours resulted in a smaller mass of iron per cell compared to other studies (30, 81, 83) but the linear relationship between SPIO exposure and intracellular iron content of the current study (Table 1) is similar to those previous efforts. For example, Heyn et al. reported intracellular iron uptake as low as 1 pg/cells when incubating with a 3 µg/mL iron concentration for 24 hours for a human pro-monocytic cell line differentiated into macrophages (83). Even for the relatively lower intracellular iron levels measured here, the detectability of labeled Bv2 cells are comparable to these previous studies conducted, especially for T$_2^*$-weighted sequences acquired at the higher fields of this study.

With respect to longitudinal relaxation, data suggests that neither increasing iron loadings nor cell count had any impact on T$_1$ values at either 11.75 or 21.1 T. In fact, there was no overall statistical difference in longitudinal relaxation between fields, except for the lowest cell count studied. These findings underscore the minimal impact that transfected SPIOs have on T$_1$ relaxation even with increasing field.

In a similar fashion, transverse relaxation demonstrated only a slight correlation with increasing iron loading or cell count. As a function of iron loading per cell, both magnetic fields displayed increasing trends of T$_2$ relaxation but only displayed statistical differences between the highest and lowest loadings, with largely no significant differences between fields. Likewise, T$_2$ relaxation displayed an increasing trend with cell count but significance was only achieved at the
lower field and between the highest and lowest cell counts. Again, with largely no differences between fields with respect to cell count, it would appear that $T_2$ relaxation was not enhanced by the higher field. More importantly, $T_2$ mechanisms could reliably predict only the extremes for either SPIO loading or cell count. Additionally, SPIO labeling displayed no benefit at 21.1 T over 11.75 T; in fact, with respect to cell count predictions, the higher field proved detrimental. Therefore, the use of SPIOs as an intracellular label for ultra high field applications would appear to be somewhat limited with respect to $T_2$.

As indicated by GRE image contrast, the more sensitive mechanism may be $T_2^*$, which better represents the impact of iron’s paramagnetic susceptibility on the induced contrast. Qualitatively, the $T_2^*$-weighted contrast of 3D GRE images (Figure 4) demonstrates sensitivities to both initial iron loading and increased cell count with respect to SPIO-induced hypointensities. As shown in Figure 8, GRE signal loss is more sensitive to the higher field strength comparing iron loadings but with no added benefit in distinguishing between different cell counts. Figure 8a illustrates a large relative signal difference between the two fields, but the $T_2^*$-weighted signal changes with increasing masses of internalized iron remain remarkably constant. However, $T_2^*$ contrast could only be used to quantify changes between the extreme iron exposures, and only at 11.75 T. As shown in Figure 8b, there are no differences in $T_2^*$-weighted signal between the fields, and the 21.1-T field provides only slight benefits, namely reduced standard deviations and significant differences between vastly different cell counts. These findings suggest that, although the use of SPIOs at the higher field may increase detectability with respect to $T_2^*$, the high field provides either the same or even reduced quantifiable metrics with regard to either iron loading or SPIO-labeled cell counts.

Two recent studies provide somewhat conflicting findings, both to themselves and the current effort. Interestingly, both of these previous studies were primarily focused on demonstrating the significantly reduced contrast enhancement evident between SPIO agents in solution and SPIOs transfected into phagocytotic cells. Working at clinical field strengths of 1.5 and 3 T and using ultra-small SPIO particles similar to those applied in the current effort, Simon and colleagues (84) transfected human monocytes with 100 µg Fe/mL to achieve a labeling of 0.7 pg/cell. Rather than conducting an exposure dose study, Simon et al. performed a cell dilution experiment with final cell counts ranging from $3.12 \times 10^6$ to $2.5 \times 10^7$ cells. For these samples, they found largely linear $R_1$ and $R_2$ relaxation rates (relative to unlabeled cells) as a
function of iron concentration, as well as significantly lower relative relaxivities for transfected SPIOs over bulk-solution SPIOs. Between fields, the intracellular relaxivities for $r_1$ remained nearly identical, while for intracellular $r_2$, the relaxivity increased by over 57% at 3 T compared to 1.5 T. In a higher field study conducted between 7 and 17.6 T, Klug and colleagues (82) evaluated experimental SPIO and commercial nanoparticles in a rat macrophage cell line by cell dilution with counts between 3.12 – 25 x $10^6$ cells. By ICP measurement, they achieved SPIO cell labeling in the range of 0.16 – 1.27 pg/cell, though labeling was highly dependent on the nanoparticle configuration. For all configurations, significant reductions in relative relaxivity were identified between bulk solution SPIOs and intracellular SPIOs. For the SPIO configuration that most closely matched the SPIO of the current study, Klug et al. demonstrated progressive reductions in relative intracellular $r_1$ and $r_2$ relaxivity with field, culminating in a factor of 3.8 reductions at 17.6 T for both mechanisms.

These contradictory results underscore some of the difficulties in conducting intracellular relaxometry studies. Alterations in experimental conditions, including cell lines, SPIO configurations, iron loading per cell, cell distributions, different pulse sequences and other factors, can alter findings significantly. The current study sought to evaluate both initial SPIO exposure (and subsequent differences in iron loading per cell) as well as alteration in cell counts; these studies are fundamentally different in that the accumulated iron within the cell and the homogeneous distribution of labeled cells within a given medium influence different exchanging pools of water (e.g. intra- versus extracellular). Clearly, both factors impact relaxivity because $T_1$ relaxation mechanisms are directly dependent on the immediate interaction of the available bulk water pool with the SPIO while $T_2$ relaxation is more dependent on particle size, intracellular aggregation, susceptibility differences between water and the SPIO, and water diffusion within the SPIO dephasing field. Furthermore, the values reported here for $R_1$ and $R_2$ are absolute numbers that are not referenced to the unlabeled cell relaxation values. The SPIO exposure and cell count studies presented here demonstrate largely equivalent relaxivities between 11.75 and 21.1 T, with only slight differences in absolute values. Given that iron is fully saturated at both 11.75 and 21.1 T so that susceptibility perturbations should be constant between the fields, the cell count findings are very consistent with dephasing due to the diffusion of a classical incoherent spin in an inhomogeneous field (78-84). Further, consistent with incoherent spin dephasing, the slight increase in $r_2$ relaxivity with SPIO exposure, particularly at 21.1 T, can be
explained by the larger sized aggregated SPIO particles induced within endosomes as a result of increased SPIO loading. Therefore, in agreement with both previous theoretical and experimental findings, the current results would be consistent with Simon et al., though the increases in $r_2$ relaxivity seen at 3 T over 1.5 T would appear to be significantly curtailed at much higher field strengths. Meanwhile, Klug et al. hypothesized that the possible quantum mechanical contribution of outer sphere relaxation theory may impart field dependence, subjugating the field independent of incoherent spin dephasing, to explain their relaxivity decreases with field. However, the authors also state that the observed decrease in $r_2$ relaxivity with field is stronger than what can be explained theoretically, even accounting for outer sphere relaxation. One potential explanation for these discrepancies may be differences in the intracellular SPIO loading of cells. For the previous study, Klug et al. achieved only 0.16 pg/cell labeling for the SPIO agent most similar to that used in the current study. The current study achieved 0.83 pg/cell loading for the cell count experiment. This factor of five difference in intracellular iron and intracellular compartmentalization should induce differences in the water exchanging environments between the studies that impact the contributions of incoherent dephasing and any other relaxation mechanism. Clearly, the current study seems to indicate that classical incoherent spin dephasing is dominant.

Neither Simon et al. nor Klug et al. evaluated the impact of SPIO on $T_2^*$ relaxation. In agreement with Klug et al., the current study was unable to quantify $R_2^*$ or $r_2^*$ relaxivity convincingly between the two high field systems due to technical difficulties and differences in field homogeneity. $T_2^*$ is notoriously difficult to measure both in vitro and in vivo because of these potential background perturbations, becoming particularly evident between repeated measures of cellular samples. However, the current study was able to assess with certainty the relative $T_2^*$-weighted contrast imparted by SPIO-labeled cells. As mentioned previously, these evaluations—as well as future attempts to better quantify $T_2^*$ relaxation—may prove more fruitful at higher fields. Through intensity comparisons made between layers, it is evident that the field dependence of $T_2^*$ contrast as a function of cell count is minimal, a comforting finding given the potential application of high field magnets to in vivo implantation studies, but that the iron content per cell and intracellular compartmentalization of iron display some field dependence. These findings support the dominance of incoherent spin dephasing, and offer a line of future inquiry for theoretical and experimental evaluations.
In conclusion, this study demonstrates that Bv2 cells can endocytose SPIOs without external transfection by means of LPS stimulation and without any impact on cellular viability, which is promising for in vivo animal models of neurodegenerative diseases. However, there were few added benefits of using SPIOs at high field beyond cell tracking. Results demonstrated little difference between the $R_1$ or $R_2$ contrast enhancement and relaxation of SPIO-labeled Bv2 cells at 11.75 and 21.1 T. However, susceptibility-based contrast mechanisms proved more beneficial for the higher field strength of 21.1 T, developing contrast for $T_2^*$-weighted sequences. Therefore, the use of SPIOs as intracellular labeling agents displayed only limited benefits at ultra-high fields beyond the ability to identify labeled cells. Among available contrast agents, SPIOs have the disadvantage of inducing severe susceptibility distortions at higher fields that can mask signals from the immediate surroundings, crucial for image interpretation. This study emphasizes the need of new MRI contrast agents for high magnetic fields that have increased relaxivity and functionality.

2.5.2 hMSC

This study shows that it is possible to label and detect hMSCs during a longer time period in a tissue mimicking substances for future in vivo cell tracking. Data suggest that hMSCs readily engulf SPIOs once exposed to the particles in the culture medium and appear to have a dose dependent uptake as seen in both MR and Prussian blue images, without any affect on proliferation as determined by MTT (Figure 10). This data also reveals a reduction in intracellular iron. This reduction is likely due to the dilution of the particles during the presumed two cell divisions occurring over the 14 day time period. The intracellular iron would assumed to be divided between the two daughter cells, however, the division of the iron is most likely not symmetric but asymmetric as seen with neural stem cells, potentially limiting long term tracking, especially with fast proliferating cell lines (85). HMSCs have previously been labeled with SPIOs with or without transfection agents. For examples SPIOs conjugated with poly-L-lysine, protamine sulfate (28) or the HIV-Tat peptide (29) have been used. Bulte et al. (30) showed that it was possible to track stem cells in vivo with a non-specific targeting, magnetodendrimer with high affinity for the cell membrane. Specific targeting can be performed by modifying the SPIOs with cell-specific receptors, such as antibodies (21, 31-33). However, as shown in this and other studies, the usage of a transfection agent or CPP is unnecessary for the uptake of SPIOs. Hsiao et
al. (47) reported that hMSCs, exposed to 100 µg/mL of iron, up took 23 pg/cell after 24 hours, and were detectable in a 1.5-T scanner without any affect on viability, proliferation or differentiation

This initial data show the potential of utilizing hMSCs not only with SPIO particles but with other particles optimized for high field MRI. The dilution of particles could be a limited factor in long-term tracking and detection in vivo. However, utilizing higher field strength could potentially prolong detectability by the increased sensitivity.
CHAPTER THREE

INTRACELLULAR BIMODAL CONTRAST AGENT

This chapter involves the conjugation of a fluorescent InP/ZnS Quantum Dot (QD) with a dysprosium (Dy) MRI contrast agent. This type of contrast agent is a novel approach that utilizes the high field relaxation properties of Dy as a MRI contrast agent and in the same time have the benefit of fluorescence by the QD. This chapter will describe the development of this bimodal intracellular QD-CAAKA-DOTA-Dy agent that shows superior MR contrast in comparison to readily available contrast agents for high fields.

3.1 Introduction

The focus of most clinical contrast agents has been the utilization of iron oxide or gadolinium compounds (21, 26, 70-74), motivated by the high sensitivity of these agents and well known chemistry involved in their fabrication. However, these particles may not be as beneficial at higher fields (>7 T). Most conventional iron oxides are known to saturate at field strengths less than 1 T while gadolinium chelates also prove optimal at fields below 1 T (16, 17). For example, even at clinical field strengths (1.5–3.0 T), the effectiveness of Gd is limited and drastically decreased—by as much as one-third with respect to its $T_1$ relaxivity—as field strength increases. Other paramagnetic lanthanides may offer improved performance at higher fields. In fact, for some cases, like dysprosium, relaxivity is expected to increase.

The benefit of using Dy$^{3+}$ are described in chapter one and the in depth mechanisms involved in the efficacy of Dy$^{3+}$ as a high field contrast agent (10-12), as well as the spin relaxation theory has been laid out by Freed (13) and Gillis P. et al (14).

The development of a bimodal MRI contrast agent that not only has MRI properties but also provides optical contrast through the conjugation of a quantum dot (QD) is advantageous as it allows for direct correlation between MR and histological confocal images. QDs have gained increased interest for biological imaging purposes because they provide a photostable alternative
to organic dye molecules, and can be color-tuned according to the size of the material. They have broad excitation wavelengths, large Stokes shifts and narrow emission lines when compared to commercially available organic dyes (86). The surface of the QD provides a large surface area that can be functionalized with a number of different ligands, including nucleic acids, peptides, proteins, antibodies and/or small molecule drugs (87-94).

Cd-Se QDs with paramagnetic surface ligands, usually appended with Gd-DTPA, (95-99) or direct nanocrystal doping with Mn$^{2+}$ (100, 101), have been used successfully as MRI contrast agents. However, concerns about cadmium and selenium environmental and physiological toxicity (102), as well as biocompatibility (renal clearance), biological stability and aqueous solubility, (103) make InP-based QDs a more bio- and environmentally compatible (104) alternative for bimodal contrast agent development. Although further nanotoxicology studies with InP QDs are warranted, previous studies on the InP bulk material that is routinely manufactured and used in semiconductor industries have shown efficient excretion of indium (105). Only high dose and long-term exposure to InP (out to 2 years) has resulted in pulmonary and reproductive impacts, with no subject deaths reported (106-109). Core-shell adducts of CdSe or CdS (core) and ZnS (shell) exhibit less toxic effects in intracellular (110-114) and live animal models (115, 116), and it is reasonable to believe that InP/ZnS core-shell materials would exhibit even fewer cytotoxic effects than these Cd-based QDs.

The choice of the semiconductor QD, surface ligand charge and hydrodynamic radius are vital for minimizing cell-disruptive responses and for complete clearance of QDs in live animals. Size, charge and biodegradability of the QD platform are critical to reduce immunogenic response (greatest with cationic particles), serum and cellular protein adsorption (117) (charge and hydrophobic interaction), and particle clearance (size and charge) (118). Particle hydrodynamic radii $\leq$5.5 nm, zwitterionic surfaces and biodegradable components enhanced renal clearance in a rat model of QD fate and transport (119). QDs greater than 5 nm in size have exhibited lymph node retention (120) while maintaining fluorescent stability up to 2 years (115). InP QDs have a smaller core-size compared to CdSe or CdS QDs with identical emission properties due to the band gap difference of the materials. Using the overall smaller InP/ZnS QD with an appended zwitterionic peptide and lanthanide chelator improves the biocompatibility and bimodal contrast ability of the nanomaterial.
3.2 Materials and Methods

3.2.1 CAAKA-DOTA-Dy and CAAKATat-DOTA-Dy formation

The CAAKA-DOTA-Dy and CAAKATat-DOTA-Dy conjugate was prepared by the condensation reaction of the DOTA-Dy complex to the lysine (K) of CAAKA (Cys-Ala-Ala-Lys-Ala) or one of the three available lysine in CAAKATat (Tat: Tyr-Gly-Arg-Lys-Arg-Arg-Gln-Arg-Arg-Arg) via a succimidyl ester. The DOTA-Dy complex is formed by mixing a 1:1 mole ratio of DyCl$_3$H$_2$O to DOTA (Strem Chemicals Inc, Newburyport, MA) in MOPS buffer (pH=6.5) for 3 h at 80°C. The DOTA is converted to the mono-succimidyl ester by treatment of the DOTA-Dy complex by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 4:1 mole ratio relative to DOTA) for 10 min to convert one of the carboxylate functionalities to the o-acylisourea intermediate. Treatment with N-hydroxysulfosuccinimide (Sulfo-NHS) (1.2:1 mole ratio to EDC) for 1 h produces the mono-functionalized succinimidyl ester of the DOTA-Dy complex. A 4:1 ratio of DOTA:EDC was used to ensure that only one carboxylate of the DOTA would be converted to a succimidyl ester (121). The CAAKA and CAAKATat peptides (SynBiosci Corp, Livermore, CA) were added in equimolar ratio to the original DOTA-Dy, mixed and incubated for 4 h at room temperature. An equimolar ratio of DOTA-Dy to peptide was used to ensure that only a single Lys residue would be labeled in both CAAKA and CAAKATat. The resultant complex was purified by HPLC (Jupiter-C18; Phenomenex Inc, Torrance, CA) using a 0-20% linear gradient of 100% CH$_3$CN, and the complex was lyophilized for later use.

3.2.2 InP-ZnS core-shell nanocrystals

ZnS-capped InP core-shell nanocrystals were prepared using a microwave-assisted lyothermal synthetic technique as described previously (122). Briefly, stock solutions of In$^{3+}$ in the form of indium palmitate (InPA) and P$^{3-}$ in the form of tris(trimethylsilyl)phosphine in decane were combined in a 1:2 mole ratio in a microwave reactor. The nanocrystals were grown at 300 W, 280°C for 15 min with “active cooling” applied in a modified CEM Discover system. The microwave generated InP nanocrystals that were 3 nm in size. Formation of the core shell
was accomplished by coating isolated InP by ZnS following the lyothermal methods outlined elsewhere (123-125). The core-shell structure was prepared by dissolving the InP nanocrystals in a 1:2 (v:v) triocylamine:dodecylamine solvent mixture heated to 250 °C. The shell was formed by slow addition of a Zn:S solution generated by mixing (Me)$_2$Zn and TMS-S in tributylphosphine. The core-shell InP/ZnS (approximately 4 nm as measured by pXRD using Scherrer analysis) was isolated by treatment with toluene followed by MeOH precipitation.

3.2.3 CAAKA-DOTA-Dy or CAAKATat-DOTA-Dy passivated ZnS/InP

Formation of the CAAKA-DOTA-Dy passivated InP/ZnS was achieved by modification of a standard method (126). Briefly, CAAKA-DOTA-Dy (1 mg) dissolved in 200-μL dimethylformamide (DMF) (5 mg/mL final peptide concentration) was added to a solution composed of 2-mg InP/ZnS dissolved in 2 mL of CH$_2$Cl$_2$:DMF (9:1, v:v). The sample was stirred for 1 h, and a solid pellet was collected by centrifugation after washing with CH$_2$Cl$_2$ and DMF. The sample was dissolved in Milli-Q water (18.3 MΩ·cm) and dialyzed (3000 -MW cutoff) in 20-mM phosphate buffer with 50-mM NaCl (pH = 7.0) to remove residual solvent and peptide.

3.2.4 X-ray fluorescence (XRF)

Elemental composition measurements for In$^{3+}$ and Dy$^{3+}$ were carried out in an Oxford Instruments ED2000 XRF spectrometer with a Cu-Kα source. For the standard XRF measurement, a 1-mg powder sample of InP/ZnS-CAAKA-DOTA-Dy was dissolved in 2 mL of fuming nitric acid (90% HNO$_3$) heated to 70°C until the sample was dry. The dry sample was dissolved in 2 mL of 2% HNO$_3$ and diluted to a 5-mL total volume in a volumetric flask using 2% HNO$_3$. Calibration curves were generated using commercially prepared 1000-ppm Dy$^{3+}$ and In$^{3+}$ elemental standards in 2% HNO$_3$, which results in accuracies of ±0.55 ppm for Dy$^{3+}$ and ±0.95 ppm for In$^{3+}$. Three independent samples were measured against a 0.2-M nitric acid control to determine the Dy:In mole ratio.
3.2.5 Cell line and transfection

Cells used in this study were Chinese Hamster Ovary (CHO) cells. They were grown at 37 °C and 5% CO\textsubscript{2} in Dulbecco’s Modified Eagle Medium (DMEM, Gibco Invitrogen Corp, Carlsbad, CA) supplemented with 10% non-essential amino acids, 10% cosmic calf serum, 1% antibiotics/antimycotics and 0.1% gentamicin. Cells were grown to approximately 90% confluence before any treatment. They were harvested by removing DMEM and washed using Tris-buffered saline (TBS). Trypsin (TrypLE, Invitrogen Corp, Carlsbad, CA) was used to detach cells from the plate. Cells were re-suspended in media for cell count and viability measurement by a trypan blue exclusion assay on a Cedex HiRes Cell Analyzer (Roche Innovatis AG, Malvern, PA) to measure cell membrane integrity. No evidence of cytotoxicity was observed with contrast agent delivery compared to controls. For all samples, the cell viability after agent transfection was measured at greater than 90% compared to control cells using this technique. In addition, viability measurements on QD-CAAKA and QD-CAAKATat configurations were made with an Invitrogen Apoptosis/Necrosis kit based on annexin V and Alexa Fluor 488 dye (Invitrogen, Carlsbad, CA). When measured with flow cytometry, CHO cells revealed no viability change compared to control (Figure 12).

![Figure 12](image-url)

**Figure 12**: Flow cytometry viability data assay on CHO cells based on annexin V and Alexa Fluor® 488 dye (Invitrogen, Carlsbad, CA). Cells were transfected for 24 h with the respective QD conjugation. A) Unlabeled CHO cells (Control), B) CHO cells transfected with 10-nM InP/ZnS-CAAKA for 24 h and C) CHO cells transfected with 10-nM InP/ZnS-Tat for 24 h.
3.2.6 Transfection with QD-CAAKA-DOTA-Dy

To achieve nanocrystal transfection, 50-µg of InP/ZnS QDs were mixed with 10-µL of Lipofectamine2000 transfecting agent (Invitrogen, Corp, Carlsbad, CA) for 20 min prior to addition to the cell samples. Lipofectamine utilizes cell cationic liposome for cell transfection, and internalization of contrast agent occurs by endocytosis through clathrin-mediated pathways (127). Cells grown in DMEM supplemented with non-essential amino acids were plated in 10-cm² sample dishes and transfected with the InP/ZnS-Lipofectamine2000 at 90% confluence (1.8x10⁶ CHO cells) following the manufacturer’s instructions. Samples were allowed to incubate for 24 h, washed with TBS, trypsinized with TrypLE and finally re-suspended in media.

3.2.7 Transfection with QD-CAAKATat-DOTA-Dy

CHO cell transfection was mediated by the HIV-Tat protein under conditions similar to the above transfection protocol but without the use of a separate transfection agent. Briefly, 50 µg of QD-CAAKATat-DOTA-Dy suspended in DMEM supplemented with non-essential amino acids was added directly to 1.8x10⁶ CHO cells (90% confluence) grown in a 10-cm² plate. Cells were incubated for 24 h, washed with TBS, trypsinized and re-suspended in media prior to MRI evaluation.

3.2.8 Sample preparation

MRI measurements were carried out with contrast agents in solution and agarose or with CHO cells suspended in agarose. Solution and agarose samples without cells were loaded in separate capillary tubes sealed with wax and imaged simultaneously. Solutions were used to evaluate the effectiveness of different agent configurations in conjugation to either the CAAKA or Tat peptides, Dy³⁺ and the QD. To measure the relaxivity of the QD-Dy agent in a tissue mimicking phantom, agarose gels were prepared in separate capillary tubes with differing masses of agent. For intracellular labeling, agarose cell layer samples were formed by mixing an equal volume of ~150,000 cells in media with a 2% low-temperature agarose (PAV2111, VWR, Suwannee, GA) to form a 1% agarose-cell final concentration.

The suspended cells were layered in a 10-mm NMR tube (Wilmad GlassLab, Buena, NJ) with a 1% agarose layer separating the cell layers. The tissue phantom also consisted of normal, untreated CHO cells to serve as a control. The remaining layers constituted a stepwise evaluation.
of the agent incubated with the CHO cells. In this setup, each of the components of the QD-CAAKA-DOTA-Dy agent was investigated for its impact on contrast enhancement and relaxation measurements. Under these conditions, the complete agent, namely the full bimodal nanoparticle consisting of QD-CAAKA-DOTA-Dy, was compared to the incomplete configurations, namely the stepwise buildup of the full agent as shown in Figure 13. Four cell samples were scanned in this fashion. Additionally, relaxivity experiments on the complete nanoparticle agent were conducted by adding QD-CAAKA-DOTA-Dy to separate cell dishes at masses of 0, 0.5, 5.0, 50 and 500 µg of QD per specimen. These samples were layered in 1% agarose as described above.

![Figure 13: CHO cell layers immobilized in agarose to evaluate MR contrast in a 10-mm NMR tube. Agarose gel was used at the bottom and as a spacer in between layers of CHO cells each transfected with a unique sample. The hypointense layer corresponds to the full bimodal contrast agent, QD-CAAKA-DOTA-Dy.](image)

3.2.9 MRI measurements

MR images were acquired on a 21.1-T, 900-MHz vertical magnet built at the National High Magnetic Field Laboratory (NHMFL) (8). The Ultra-Widebore (UWB) magnet has an inner diameter of 105 mm, and the system is equipped with a Bruker Avance console with Micro2.5 microimaging gradients (Bruker Corp, Billerica, MA). A 10-mm Bruker birdcage coil was used for imaging all samples. MRI parameters were optimized to image the bimodal contrast agent in agarose and solution depending on the experiment. Typical sequences parameters used
included: matrix = 128x128, BW = 75k Hz. FOV = 1.9x1.0 cm and slice thickness = 1.0 mm. For T<sub>1</sub> and T<sub>2</sub> measurements, a single slice 2D spin echo (SE) sequence was applied by varying the echo time (TE = 10-300 ms) or repetition time (TR = 25-15000 ms), respectively. T<sub>2</sub>* contrast was acquired with a 2D gradient-recalled echo (GRE) sequence with varying TEs (5-85 ms). High resolution 3D GRE images were acquired with TE=5, TR=100 ms and BW = 55 kHz. The acquisition matrix was adjusted to accommodate the FOV to achieve an isotropic resolution of 50 µm. All acquisitions were performed at 23 °C.

3.2.10 MR image analysis

Magnitude images were analyzed using Bruker ParaVision 3.1.2. Regions of Interest (ROIs) were drawn to cover each separate solution, gel or cell layer sample. Signal intensity data were analyzed in SigmaPlot 7.101 (SPSS, Inc., Chicago, IL). Using a normalized noise baseline, data was fitted to a three-parameter exponential decay for T<sub>2</sub> and T<sub>2</sub>* values and a three-parameter exponential growth for T<sub>1</sub>. An ROI also was drawn outside of the sample to measure the mean noise baseline of the magnitude images.

3.2.11 Confocal microscopy

Images of CHO cells incubated with QDs were acquired with an inverted Nikon TE2000-E2 Eclipse C1si confocal laser scanning microscope (CLSM) from Nikon Instruments Inc. (Melville, NY,) equipped with a Nikon CFI Plan Apochromat 40X objective (NA 0.95, 0.14-mm WD) and a transmitted light detector. The sample was excited with a 404 violet laser and detected using a 102.6-µm pinhole and the following emission filters: 515/30 and 605/75.

3.2.12 Statistics

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS, Inc. Chicago, IL). ANOVA statistical analysis was performed for T<sub>2</sub> and T<sub>1</sub> relaxation for the different conjugations of the contrast agent, and significant differences between samples were determined using the Tukey’s Honestly Significant Differences (HSD) post hoc test. Data was determined to be statistically significant at p ≤ 0.07.
3.3 Results

3.3.1 InP/ZnS fluorescence, Excitation/Emission profiles, TEM verification and XRF analysis

Figure 14a shows the pXRD data of the InP/ZnS nanocrystal while a TEM image is shown in Figure 14b. The pXRD data of the InP/ZnS QD displays peaks at 28, 47 and 56 degrees corresponding to ZnS. No peaks are evident for InP because the ZnS shell completely isolated this inner core. Therefore, the x-ray diffraction pattern confirms the intended core/shell configuration, as seen in TEM. Figure 14c illustrates the absorbance spectra of the InP/ZnS (core/shell) at 525 nm of the nanoparticle without Dy\(^{3+}\) while the Figure 14d displays the photoluminescence of the InP/ZnS-CAAKA-DOTA QD with and without Dy\(^{3+}\). As shown by these measurements, the addition of the Dy\(^{3+}\) causes only a slight decrease in photoluminescence of about 20% and a wavelength shift of 20 nm.

The mole ratio of Dy\(^{3+}\) on the InP/ZnS QD was determined through XRF analysis of three independent samples, resulting in 16.0 ± 0.6 ppm Dy\(^{3+}\) (or 0.1 mmol/kg) per 27.9 ± 0.95 ppm In\(^{3+}\) (or 0.243 mmol/kg) a mole ratio of 0.411. Using this ratio and the fundamental makeup of the QD core (201 In\(^{3+}\) and 216 P\(^{3-}\) atoms per 2.7-nm InP as determined by CrystalMaker Software Ltd., Oxfordshire, UK), 82 Dy\(^{3+}\) per InP core were measured.
Figure 14 a: pXRD of InP/ZnS nanocrystal. Peaks at 28, 47 and 56 degrees correspond to ZnS. The absence of the InP peaks and presence of only ZnS peaks in the x-ray diffraction pattern confirm the core/shell configuration and complete coverage of InP b: TEM of InP/ZnS nanocrystal (~4 nm) and c: Absorbance spectra for InP/ZnS (core/shell) nanoparticle without Dy$^{3+}$ d: Excitation/Emission Spectra of (—) InP/ZnS-CAAKA-DOTA (no Dy) and (- -) InP/ZnS-CAAKA-DOTA-Dy. The addition of the Dy$^{3+}$ ion causes a decrease of ~20% of the photoluminescence intensity.
3.3.2 Bimodal contrast agent in solution

Different conjugations of the proposed agent were imaged in solutions to assess contrast enhancement. Samples with the conjugating peptide CAAKA as well as with the Tat peptide were investigated. In each instance, the same mass (50 µg) of agent was added to the same volume of nanopure water, resulting in different moles of Dy$^{3+}$ for each solution (Table 5 and Figure 15). Solutions containing Dy$^{3+}$ provided contrast enhancement at 21.1 T. Neither the peptides nor the QD provided contrast with respect to $T_1$, $T_2$ or $T_2^*$ in the absence of dysprosium. Furthermore, while the Tat and CAAKA conjugation with Dy-DOTA displayed reduced $T_1$ and $T_2$ relaxation, the QD configurations of these peptides with Dy-DOTA presented more significant relaxation enhancement and chemical shift, even though the molar concentration of Dy$^{3+}$ in the QD agents was approximately 25% lower than the chelates alone in solution.

Table 5: Solution samples of different CA configurations with relaxation times as shown in Figure 15.

<table>
<thead>
<tr>
<th>CA configuration (50 µg per sample)</th>
<th>Nanomoles of Dy$^{3+}$ per sample</th>
<th>$T_1$ (ms)</th>
<th>$T_2$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CAAKA-DOTA</td>
<td>0</td>
<td>2757.1</td>
<td>508.2</td>
</tr>
<tr>
<td>2. Tat-DOTA</td>
<td>0</td>
<td>2682.5</td>
<td>492.3</td>
</tr>
<tr>
<td>3. QD-Tat</td>
<td>0</td>
<td>2665.1</td>
<td>480.8</td>
</tr>
<tr>
<td>4. Tat-DOTA-Dy</td>
<td>16.1</td>
<td>561.0</td>
<td>316.6</td>
</tr>
<tr>
<td>5. CAAKA-DOTA-Dy</td>
<td>47.6</td>
<td>521.8</td>
<td>237.0</td>
</tr>
<tr>
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<td>35.0</td>
<td>69.0</td>
<td>42.9</td>
</tr>
<tr>
<td>7. QD-Tat-DOTA-Dy</td>
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</tbody>
</table>
For a realistic assessment of relaxivity in tissue, the QD-CAAKA-DOTA-Dy agent was evaluated in an agarose preparation by incrementing the mass of agent in individual capillary tubes (inset of Figure 16b). With increasing QD mass, an expected increase in relaxation was observed as illustrated in Figure 16 and Table 6. A quadratic regression was performed to calculate the respective relaxivity values in a non-uniform sample as indicated by Clark et al. (128). The $r_2$ relaxivity was calculated to be 57.4 mM$^{-1}$s$^{-1}$, while the $r_1$ relaxivity was substantially less at 0.08 mM$^{-1}$s$^{-1}$. As seen with other intracellular contrast agents, the $T_2$ relaxing property of the QD-Dy dominates the $T_1$ effects of the agent in this agarose tissue phantom (72, 79, 129). This trend also was apparent in in vitro experiments.

**Figure 15**: $T_1$- (TE/TR = 7/50 ms) and $T_2$- (TE/TR = 106/5000 ms) weighted SE images illustrating different conjugations of CA in solution as outlined in Table 1. c: Illustration of tube position with numbers referring to Table 5.
**Figure 16 a**: illustrates the increase in $T_1$ relaxation with increasing $\text{Dy}^{3+}$ concentration. Quadratic regression yields: $r_1 (\text{mM}^{-1} \text{s}^{-1}) = 0.36 + 0.08[\text{Dy}] - 0.01[\text{Dy}]^2$ ($R^2 = 0.9742$). **b**: $r_2$ relaxivity graph illustrates the increase in $T_2$ relaxation with increasing $\text{Dy}^{3+}$ concentration. Quadratic regression yields: $r_2 (\text{mM}^{-1} \text{s}^{-1}) = 5.74 + 57.4[\text{Dy}] - 9.60[\text{Dy}]^2$ ($R^2 = 0.9951$). **INSET**: $T_2$-weighted SE image (TE/TR = 5/5000 ms) of increasing QD-CAAKA-DOTA-Dy masses immobilized in agarose.
Table 6: \( T_1 \), \( T_2 \) and \( T_2^* \) relaxation times for incremented QD masses in 1% agarose as shown in Figure 13.

<table>
<thead>
<tr>
<th>QD-CAAKA-DOTA-Dy mass (µg)</th>
<th>Effective Dy(^{3+}) Concentration (mM)</th>
<th>( T_1 ) (ms)</th>
<th>( T_2 ) (ms)</th>
<th>( T_2^* ) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.0035</td>
<td>2759.7</td>
<td>177.3</td>
<td>---</td>
</tr>
<tr>
<td>5</td>
<td>0.035</td>
<td>2812.9</td>
<td>112.8</td>
<td>21.9</td>
</tr>
<tr>
<td>50</td>
<td>0.35</td>
<td>2486.7</td>
<td>44.4</td>
<td>12.1</td>
</tr>
<tr>
<td>75</td>
<td>0.525</td>
<td>2460.0</td>
<td>30.9</td>
<td>10.9</td>
</tr>
<tr>
<td>150</td>
<td>1.05</td>
<td>2302.5</td>
<td>16.6</td>
<td>3.8</td>
</tr>
<tr>
<td>250</td>
<td>1.75</td>
<td>2069.5</td>
<td>13.6</td>
<td>3.3</td>
</tr>
<tr>
<td>350</td>
<td>2.45</td>
<td>2069.5</td>
<td>11.2</td>
<td>2.1</td>
</tr>
<tr>
<td>500</td>
<td>3.5</td>
<td>1899.2</td>
<td>11.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

3.3.3 Intracellular evaluation of bimodal contrast agent

CHO cells were incubated with a stepwise build-up of the QD-CAAKA-DOTA-Dy bimodal agent for evaluation of the intracellular contrast. Labeled cells with different configurations of the CA were suspended in 1% agarose as described in Figure 13. In Figures 17b and 17c, \( T_2 \)-weighted SE and GRE images, respectively, of CHO cells immobilized in agarose are shown. The second layer from the top (in both images), containing CHO cells transfected with the complete QD-CAAKA-DOTA-Dy agent, is the only layer showing \( T_2/T_2^* \) contrast, with a \( T_2 \) value of 72.2 ms (Table 7). The \( T_2 \) relaxation in this layer is approximately \( \frac{3}{4} \) of that observed in all other cell/agarose layers, with Table 7 displaying the quantitatively measured \( T_1 \) and \( T_2 \) relaxation of the different conjugations. ANOVA one-way analysis shows significance between groups at a \( p<0.007 \) for \( T_2 \) relaxation. Tukey’s HSD post hoc test verified significance in CHO cells labeled with the complete QD-CAAKA-DOTA-Dy agent compared to all other configurations (\( p<0.07 \)). This layer also shows a reduction in average \( T_2^* \) (= 46.6 ms) compared to agarose (\( T_2^* = 131.2 \) ms) and all other agent configurations at (\( T_2^* = 62 \) ms).
the $T_2^*$-weighted GRE images, the labeled cells demonstrate an even distribution throughout the agarose layer.

An ANOVA one-way analysis also shows significance between groups at a $p<0.001$ for $T_1$ relaxation. In post-hoc analysis using Tukey’s HSD test for significance, the $T_1$ of the unlabeled CHO cells was found to be significantly different from the background agarose and all configurations that utilized either QDs or Dy$^{3+}$ ions; however, there was no significant difference between any of the agent configurations with respect to $T_1$, suggesting that the complete agent is not performing as a significant intracellular $T_1$ agent in this model cellular system. Rather, it is likely that the process of transfection-induced endocytosis and subsequent vesicle formation has a slight impact on the average $T_1$ of all labeled cells, regardless of the endocytosed material. As a result, the complete QD-CAAKA-DOTA-Dy configuration is functioning primarily as a $T_2/T_2^*$ agent when used intracellularly, providing a sufficient payload of Dy$^{3+}$ to generate MRI contrast.

Table 7: $T_1$ and $T_2$ relaxation data from CHO cells labeled with a stepwise build-up of the QD-CAAKA-DOTA-Dy agent as shown in Figure 13.

<table>
<thead>
<tr>
<th>CHO cells labeled with Bimodal CA</th>
<th>Number of Samples</th>
<th>$T_1$ (ms)</th>
<th>$T_2$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QD-PEPTIDE</td>
<td>4</td>
<td>3094.8 ± 64.2$^a$</td>
<td>99.7 ± 15.8$^b$</td>
</tr>
<tr>
<td>QD-CAAKA-DOTA-Dy</td>
<td>5</td>
<td>3022.7 ± 52.3$^a$</td>
<td>72.2 ± 10.3</td>
</tr>
<tr>
<td>CAAKA-DOTA-Dy</td>
<td>4</td>
<td>3020.0 ± 40.7$^a$</td>
<td>101.3 ± 13.7$^b$</td>
</tr>
<tr>
<td>CAAKA-DOTA</td>
<td>3</td>
<td>3190.4 ± 60.9</td>
<td>106.4 ± 14.7$^b$</td>
</tr>
<tr>
<td>Unlabeled CHO cells</td>
<td>5</td>
<td>3453.8 ± 245.8</td>
<td>101.0 ± 16.9$^b$</td>
</tr>
<tr>
<td>Agarose</td>
<td>5</td>
<td>3115.53 ± 165.5$^a$</td>
<td>107.5 ± 9.9$^b$</td>
</tr>
</tbody>
</table>

$^a$ Significantly different from $T_1$ of unlabeled CHO cells as assessed by Tukey’s HSD ($p < 0.07$)

$^b$ Significantly different from $T_2$ of QD-CAAKA-DOTA-Dy as assessed by Tukey’s HSD ($p < 0.07$)
A similar cell-agarose setup was instituted with CHO cells incubated with QD-CAAKA-DOTA-Dy at increasing QD masses. Not surprisingly, the amount of QD-CAAKA-DOTA-Dy delivered to the cell results in a substantially greater transverse relaxation. Minimal $T_2$ values were achieved with an incubation of 500 $\mu$g, generating a $T_2$ of 19.0 ms, while CHO cells without any loading displayed an average $T_2$ of 101.0 ms. The 50-$\mu$g QD-CAAKA-DOTA-Dy incubation shown in Table 8 yielded a $T_2$ of 62.4 ms, which approximates the previous results of Figure 17 and Table 7.
Using the fluorescent properties of the QD, optical images were acquired to verify uptake of the complete agent, identify intracellular localization of the agent and assess the impact of MRI visibility on optical performance (Figure 18). Compared to background fluorescence (Figure 18a), a bright yellow emission (550 nm) was observed from an even distribution of CHO cells (Figure 18b and 18c). With counterstaining for nuclear and cell membrane proteins, specific bright yellow fluorescent regions internal to the cell (Figure 18d and 18e) verify the internalization of the agent into endosomes or lysosomes. Furthermore, these images demonstrate the localization of agent-loaded vesicles to the perinuclear region, with a generally higher concentration of fluorescing nanoparticles achieved through the use of the Tat peptide (Figure 18e).

Table 8: $T_1$ and $T_2$ relaxation data from CHO cells transfected with different masses of QD-CAAKA-DOTA-Dy

<table>
<thead>
<tr>
<th>QD mass ($\mu$g)</th>
<th>$T_1$ (ms)</th>
<th>$T_2$ (ms)</th>
<th>$T_2^*$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>3117.6</td>
<td>19.0</td>
<td>7.4</td>
</tr>
<tr>
<td>50</td>
<td>3102.6</td>
<td>62.4</td>
<td>31.9</td>
</tr>
<tr>
<td>5</td>
<td>3127.1</td>
<td>106.5</td>
<td>53.7</td>
</tr>
<tr>
<td>0.5</td>
<td>3236.0</td>
<td>98.7</td>
<td>89.2</td>
</tr>
<tr>
<td>Agarose</td>
<td>3385.5</td>
<td>104.6</td>
<td>69.0</td>
</tr>
</tbody>
</table>

Using the fluorescent properties of the QD, optical images were acquired to verify uptake of the complete agent, identify intracellular localization of the agent and assess the impact of MRI visibility on optical performance (Figure 18). Compared to background fluorescence (Figure 18a), a bright yellow emission (550 nm) was observed from an even distribution of CHO cells (Figure 18b and 18c). With counterstaining for nuclear and cell membrane proteins, specific bright yellow fluorescent regions internal to the cell (Figure 18d and 18e) verify the internalization of the agent into endosomes or lysosomes. Furthermore, these images demonstrate the localization of agent-loaded vesicles to the perinuclear region, with a generally higher concentration of fluorescing nanoparticles achieved through the use of the Tat peptide (Figure 18e).
3.3.4 Self-transfecting configuration

The QD-CAAKATat-DOTA-Dy configuration has been imaged with and without QDs in solution and 1% agarose. The $T_1$ contrast in solution can be seen in Figure 19, where a $T_1$-weighted image shows hyperintense contrast for the QD-CAAKATat-DOTA-Dy configuration in both solution and agarose (tubes 3 and 4 in Figure 19a) with $T_1$ values of 1.27 and 1.32 s, respectively (water $T_1 = 2.96$ s). Likewise, $T_2$ measurements of the QD-CAAKATat-DOTA-Dy agent in solution and agarose yielded values of approximately 62 ms, representing a 56% decrease in $T_2$ compared to bulk water. When introduced to CHO cells, the QD-CAAKATat-
DOTA-Dy agent enhances $T_2/T_2^*$ contrast, even though $T_1$ relaxation is minimal, as was seen previously with the QD-CAAKA-DOTA-Dy configuration (Figure 19b). Notably, the CAAKATat configuration provides reduced and nearly identical $T_2$ values (Table 9) regardless of whether an external transfection agent is used to label the cell. As such, this QD-Dy formulation is capable of self-transfection to induce $T_2/T_2^*$ enhancement as an intracellular label.

**Figure 19 a:** $T_1$-weighted SE image with TE/TR = 7/150 ms. 1. CAAKATat-DOTA-Dy in solution ($T_1 = 2.02$ s; $T_2 = 307.3$ ms) 2. CAAKATat-DOTA-Dy in agarose ($T_1 = 2.03$ s; $T_2 = 65.3$ ms) 3. QD-CAAKATat-DOTA-Dy in solution ($T_1 = 1.27$ s; $T_2 = 312.8$ ms) 4. QD-CAAKATat-DOTA-Dy in agarose ($T_1 = 1.32$ s; $T_2 = 73.4$ ms). **b:** 3D GRE (TE/TR = 5/100ms) with QD-CAAKATat-DOTA-Dy labeled CHO cells immobilized in agarose layers nomenclature as Table 5. Layers 2 and 4 represent unlabeled CHO cells. From this image and the relaxation data of Table 5, $T_2$ contrast is readily apparent and dominates any $T_1$ contrast.
3.4 Discussion

The QD-CAAKATat-DOTA-Dy agent displays strong MR contrast enhancement for all relaxation mechanisms in solution. As shown by the differences between the chelated Dy$^{3+}$ and QD-Dy configurations, one of the main benefits of using a QD is that the payload of DOTA-Dy complex can be substantially increased per QD, with 82 Dy$^{3+}$ per InP/ZnS QD as determined by XRF measurements. Even more importantly, the improved contrast enhancement of the dysprosium agent with conjugation to the QD may result from more direct water access to the DOTA-Dy chelate, particularly in the case of the Tat peptide. As shown in Table 5, $T_1$ and $T_2$ values decrease significantly for the QD configurations compared to other formulations even though the total mass of Dy$^{3+}$ actually decreases. For the self-transfecting configuration, the longer polypeptide chain of the Tat molecule, with one end tethered to the QD surface, may offer even more water accessibility to Dy-DOTA as demonstrated by the reduced $T_1$ and $T_2$ values for this configuration when compared to QD-CAAKA-DOTA-Dy (Table 5).

In agarose and labeled CHO cells, the dominant contrast mechanisms are $T_2$ and $T_2^*$ relaxation, with little or no $T_1$ enhancement. This trend is demonstrated in relaxivity experiments conducted in agarose, for which the $r_2$ relaxivity proved to be three orders of magnitude higher than the $r_1$ relaxivity. It also is notable that a quadratic function, which statistically provided the

<table>
<thead>
<tr>
<th>Agent</th>
<th>$T_1$ (ms)</th>
<th>$T_2$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. QD-CAAKATat-DOTA-Dy with Lipofectamine</td>
<td>2977.9</td>
<td>55.5</td>
</tr>
<tr>
<td>2 &amp; 4. Average unlabeled CHO cells</td>
<td>3088.0</td>
<td>100.5</td>
</tr>
<tr>
<td>3. QD-CAAKATat-DOTA-Dy without Lipofectamine</td>
<td>3011.8</td>
<td>55.0</td>
</tr>
<tr>
<td>Agarose</td>
<td>3051.0</td>
<td>100.9</td>
</tr>
</tbody>
</table>

Table 9: Relaxation times for CHO cells labeled with the QD-CAAKATat-DOTA-Dy agent comparing the effects of Lipofectamine usage.
best fit of the data points, was employed in these calculations because of the heterogeneity of the sample (namely simultaneous T\textsubscript{2} contributions from both the agent and the background agarose) (128) and the apparent plateau of relaxivity at high QD-CAAKA-DOTA-Dy concentrations in agarose. A similar quadratic trend has been identified in cells labeled with either gadolinium or iron oxides (79). Interestingly, T\textsubscript{1} enhancement was evident in agarose phantoms when a QD-CAAKATat-DOTA-Dy configuration was used (Figure 19). Again, this finding lends credence to the theory that the Tat peptide, likely due to its increased length, provides increased water access to the DOTA-Dy molecule.

With transfection into CHO cells, the QD-CAAKA-DOTA-Dy agent generated strong T\textsubscript{2}/T\textsubscript{2}\text{*} contrast (Figure 17). This contrast was unique to the complete agent, yielding at least a 25% reduction in T\textsubscript{2} and T\textsubscript{2}\text{*}. The payload of Dy\textsuperscript{3+} conjugated to the QD also proved critical to the transverse relaxation, as demonstrated by the lack of contrast apparent in incomplete configurations of the agent. As demonstrated by recent studies that have coupled CdSe QD with paramagnetic materials (100, 130-133), the complete In/P QD agent proved superior to isolated paramagnetic chelates and peptides by actually increasing the delivered Dy\textsuperscript{3+} and resultant MR contrast enhancement. In this respect, the QD approach may be superior to other bimodal agents that have been used to corroborate the localization of the agent either intracellularly or systemically by incorporating organic fluorescent dyes coupled singly to paramagnetic agents (134, 135).

Capitalizing on the bimodal nature of the complete nanoparticle, the intracellular localization of the agent was verified by confocal microscopy (Figure 18), indicating that the nanoparticle is assumed to be sequestered in vesicles of the perinuclear region. As in the agarose phantoms, the intracellular sequestration of the bimodal agent to endo- or liposomes results in a lack of T\textsubscript{1} contrast, even though it was apparent in solution samples. A likely explanation is that the internalization of agents in the intracellular vesicles limits water access, resulting in a saturated but relatively small pool of longitudinal relaxed water inside the vesicle (136). Known as T\textsubscript{1} quenching, this effect was expected as a result of the method of transfection used in this study, namely Lipofectamine, which induces internalization through the lyso-endosomal pathway. The low surface-to-volume ratio and limited water exchange across the endosomal membrane of the vesicle will reduce longitudinal relaxation. T\textsubscript{1} quenching has been observed by others in cell labeling efforts (72, 79), with the consensus being that a three-compartment model...
of relaxation yields high relaxation inside the agent-loaded vesicle but slow water exchange between the vesicle and cytosol to achieve only a partly $T_1$-enhanced cytosol. Interestingly, the QD-CAAKATat-DOTA-Dy agent also demonstrated $T_1$ quenching, indicating that the ultimate fate of the self-transfecting agent was vesicular sequestration. These findings were confirmed by confocal fluorescent imaging. Fortunately, transverse ($T_2$ or $T_2^*$) relaxation is not affected by agent localization because the paramagnetic perturbation induced by Dy$^{3+}$ propagates beyond the endosomes to affect intracellular water.

QD loading was assessed by incubating CHO cells with different masses of the QD-CAAKA-DOTA-Dy agent to investigate Lipofectamine-moderated uptake. The loading limit of Lipofectamine, as determined by the manufacturer, is commonly expressed per mass DNA, and the bimodal nanoparticle most likely loads cationic endosomes differently. Results demonstrated a consistent decrease in $T_2$ and $T_2^*$ with increasing incubation masses of QD agents from 0 – 500 µg (i.e. Dy$^{3+}$ loading), suggesting that QD labeling was not limited by the transfection agent and could be increased by higher exposure levels during incubation to enhance sensitivity and detectability of labeled cells (Table 8). To assess toxicity, preliminary membrane integrity studies utilizing trypan blue exclusion and an apoptosis/necrosis assay on QD-peptide conjugations (Figure 12) have shown no cellular toxicity at the concentrations used for MR imaging. More detailed toxicity studies are underway, but to date, no adverse reaction or impact on cellular viability has been identified for even the highest labeling of CHO cells with the complete bimodal agent.

Incorporation of the Tat peptide in the bimodal nanoparticle agent eliminates the need for an external transfection agent (137). Additionally, the Tat molecule attached to the CAAKA peptide creates a longer surface functionalizing peptide, which would extend the DOTA-Dy complex farther from the QD surface to improve water access and create a more favorable exchange regime. This process may be evident in agarose experiments that displayed $T_1$ enhancement for the QD-CAAKATat-DOTA-Dy agent, although a similar $T_1$ effect was not discernable in vitro (Figure 19). Previous reports indicate that exogenous Tat enters the cell by absorptive endocytosis, a common pathway for many viruses utilizing peptide binding to charged species on the cell surface (138). Tat peptide tagged with fluorescent markers normally localizes in the cytoplasmic or nuclear compartment (139); therefore, it was expected that Tat might facilitate $T_1$ enhancement. However, the apparent $T_1$ quenching and corroborative confocal
images suggest that Tat functionalized nanoparticles still are sequestered ultimately in perinuclear vesicles. It is possible that the Tat-functionalized agent may eventually accumulate inside subcellular vesicles by clathrine-mediated endocytosis (127) or macropinocytosis triggered by binding to the negatively charged membrane (140). While maintaining the self-transfecting properties of the Tat peptide, future efforts will be directed at determining which functional modifications of linking peptides can enhance T₁ contrast, possibly by either prohibiting the uptake of the agent in vesicles or affecting the release of vesicle-sequestered agents into the cytoplasm after transfection.

Other materials, such as superparamagnetic iron oxides (SPIOs) and gadolinium chelates, have a similar effect on MR relaxation (principally T₂ and T₂*) that results in a signal loss when used as intracellular labels. SPIOs have a high sensitivity, providing domains that are thermodynamically independent and (contrary to the single atom alignment of dysprosium) aligned with the B₀ field as a single unit (21, 26). SPIO alignment generates microscopic field gradients that dephase neighboring protons (20). As such, SPIOs induce a hypointense signal at the site of the agent and beyond. Particularly at high fields, however, the magnetic susceptibility perturbation induced by SPIOs can be so pronounced that signal from surrounding soft tissue can be lost, causing a “blooming” of hypointensity around the nanoparticle. This artifact effectively can reduce the information content of the MR scan by either destroying signal or masking other anatomical features (21). Likewise, gadolinium chelates have been implemented to track stem cells in vivo (7, 141), but their primary mode of action—like SPIOs—is a susceptibility perturbation that induces signal loss in surrounding water, though the magnitude of dephasing from intracellular Gd is much lower than that of SPIOs due to the comparatively reduced magnetic moment of the lanthanide.

The current bimodal agent based on a QD-CAAKA-DOTA-Dy configuration compares quite favorably to SPIOs, Gd and Mn as an intracellular label. Due to the payload delivery of 82 Dy³⁺ ions per QD, the QD-CAAKA-DOTA-Dy bimodal agent was able to provide r₁ and r₂ relaxivities of 0.08 and 57.4 mM⁻¹s⁻¹, respectively. These relaxivities were measured in a tissue relevant phantom (1% agarose) and at 21.1 T. For comparison, ferumoxides, ferumoxtrans and ferumoxsil have reported r₁ and r₂ relaxivities of 3.2-23.7 and 53.1-107 mM⁻¹s⁻¹, respectively, at 0.47 T (142) while Gd-chelates provide r₂ relaxivities in the range of 5-15 mM⁻¹s⁻¹. Other QD-based MR agents have reported r₁ and r₂ relaxivities of 14.4 and 23.04 mM⁻¹s⁻¹ for gadolinium.
conjugates (131) at 1.5 T and $r_1 = 18 \text{ mM}^{-1} \text{s}^{-1}$ for manganese conjugates at 7 T (143). It should be noted that these previously reported evaluations were conducted at significantly lower field strengths (0.47-7 T) and in mostly aqueous solutions, not the agarose tissue phantom reported here. It would be expected that both gadolinium and manganese would perform significantly worse at 21.1 T compared to a lower field strength. To investigate this field dependence between lanthanides and confirm previous reports (10-14, 16-18), the relaxivities of gadolinium and dysprosium (both in chloride form) were compared at 11.75 and 21.1 T. In agreement with literature, the relaxivity associated with dysprosium increased by 66.4% and 68.2% for $r_1$ and $r_2$, respectively, when moving from 11.75 to 21.1 T while gadolinium displayed a decrease in relaxivity of 10.0% for $r_1$ and 6.1% for $r_2$ over the same fields.

The InP/ZnS QD conjugated with DOTA-Dy provided a small nanoparticle (4 nm vs. 31 nm (131) and 4.1-4.7 nm (143)) with a minimal number of paramagnetic ions per particle (82 Dy$^{3+}$ vs. 2500 Gd$^{3+}$ (131) or 52 Mn$^{2+}$ (143)) but increased $r_2$ relaxivity by at least a factor of two over a previously reported Gd QD preparation (131) and by a factor of 20 over a clinically available Mn agent (Teslascan, GE Healthcare, Inc.). As such, the InP/ZnS QD and Dy agent at 21.1 T is very comparable if not superior, and further enhancements of peptide-DOTA-Dy binding can increase the payload and improve relaxivity, as has been accomplished with lipid bound Gd$^{3+}$ to QDs (132). Additionally, the QD-CAAKA-DOTA-Dy agent, though having a relaxivity within the range of commercial iron oxide configurations (142), provides a significantly reduced “blooming effect” compared to SPIOs, maintaining the information content of MR images.

In conclusion, a bimodal contrast agent was created using DOTA doped with Dy$^{3+}$ ions appended to the surface of an InP/ZnS QD. Applied in solutions, gels and labeled CHO cells, the complete agent demonstrated both MR contrast and fluorescence detection. This nanoparticle was designed to perform as an intracellular agent for high field applications, namely future efforts to track implanted cell lines using the highest magnetic field available for MR imaging, 21.1 T. Fluorescent imaging together with the observed T$_1$ quenching indicates that our bimodal agent is located in intracellular vesicular compartments. As such, the bimodal nanoparticle functioned primarily as a T$_2$/T$_2^*$ contrast agent when used to label cells intracellularly. Furthermore, the incorporation of Tat peptide showed effective self-transfecting properties. Using this high field optimized agent, implanted cell lines and endogenous cells (including stem
cells) potentially could be labeled to track cell migration, fate and transport for *in vivo* applications using both MRI and optical techniques. The bimodality of this contrast agent provides the opportunity to monitor cells longitudinally and at high resolution by correlating fluorescent and MR imaging.
CHAPTER FOUR

IN VIVO TRACKING OF NEUROPROGENITOR CELLS AND HMSC

Neuronal progenitor cells (NPCs) from the subventricular zone are known to migrate continuously along the rostral migratory stream (RMS) to the olfactory bulb (OB). Research suggests that these cells also can migrate to lesions outside the RMS caused by oxygen deficiency in the case of stroke or TBI. In this chapter, the utilization of iron oxides at high field to label different cell types both endogenously and exogenously will be described. In particular, the transfection of endogenous NPCs in the subventricular zone in association with a TBI as well as implanting individually exogenously labeled hMSCs, through the carotid artery. This chapter provides a cell-specific in vivo tracking of the migration to sites of neuronal injury.

4.1 Background

Neurogenesis takes place in limited places in the brain: the dentate gyrus (DG) in the hippocampus formation (HF) and the subventricular zone (SVZ). These two areas are well known to harbor cells capable of generating cells that can turn into neurons, although it has been suggested that neurogenesis can occur in the neocortex of adult primates (145) and humans (146, 147) and it is estimated to produce 30,000 cells bilaterally on a daily basis in the mouse (148). Neuroprogenitor cells in the SVZ are mitotic cells that have an ability to proliferate and give rise to terminally differentiated cells, which in contrast to stem cells have the ability to proliferate, undergo self-renewal and differentiate into neurons, astrocytes and oligodendrocytes (149, 150).

The SVZ is located next to the lateral wall of the lateral ventricle and has been proven to contain both stem-like cells as well as rapidly proliferating progenitor cells (151). In more detail, four types of cells have been identified: Type A, B, C and E. Type E cells are ependymal cells that do not become neurons but instead form the lining of the ventricles (152). Type A cells, on the other
hand, have been shown to be groups of migrating neuroblasts that are organized in a chain-like structure. Type B cells are slowly proliferating cells with a tube like structure and astrocyte characteristics. These cells also separate the SVZ from surrounding tissue. There are two variants of the Type B cell—B1 and B2—that surround Type A cells. B1 cells are located in the interface between Type A cells and the lining of the ventricles (E cells) while B2 cells are located between the striatum and chains of migrating cells (Type A). Type C cells are actively proliferating cells that are scattered through the SVZ. Their location and frequency of division suggest that Type C cells are precursors to Type A cells; Type B cells are believed to be precursors of Type C cells. These conclusions are supported by experiments performed with an antimitotic drug introduced by intracerebroventricular (ICV) injection that eliminated Type A and C cells (153). After the discontinuation of the antimitotic treatment, Type C cells reappeared first followed by Type A cells. This work suggests that the neuronal lineage starts with Type B cells with their stem-like characteristics that differentiate into Type C cells with their progenitor characteristics and then ends with Type A cells as the migrating neuroblasts that ultimately undergo a final differentiation to immature and mature neurons (151-154).

Figure 20: Illustration of the SVZ and granular layers. The left image is a coronal section through the telencephalon of an adult mouse brain showing the ventricles and the lateral position of the SVZ. The cortex (CTX), corpus callosum (CC) and striatum (STR) are also shown. The right image is an illustration of the granular layers showing the four types of cells types. Modified from Garcia-Verdugo et al 1998 (154).
The majority of progenitor cells developed in the SVZ are destined for the olfactory bulb (OB), traveling along a well-defined pathway called the rostral migratory stream (RMS). Most NPCs proliferate in the SVZ, migrate along the RMS and being to differentiate during their travel, ultimately resulting in terminally differentiated neurons incapable of indefinite self-renewal (151). Figure 21 illustrates the migration pathway and the differentiation that occurs along the way. The method of migration by the NPCs differs from progenitors developed in other areas of the brain. The migration of progenitors from the SVZ is called chain migration and is not aided by glial cells (155). Chain migration is solely executed by Type A cells that form long chains through homotypic interactions. These chains are surrounded by Type B cells that form a tubular scaffold (156). The migration is believed to be aided by a modified form of the neural cell adhesion molecule (NCAM) called PSA-NCAM. (152, 157, 158). It is thought that the polysialylated (PSA) moiety of the PSA-NCAM molecule is a regulator of cell-cell interaction allowing cells to detach and slide along each other (159). This type of migration allows for a much faster transportation of cells, and it has been shown by Wichterle et al. that they move at an average speed of 120 µm/h (155), which is about four times faster than normal migration along glial fibers.

**Figure 21:** Sagittal view of a rodent brain showing the SVZ, migration along the RMS and finally reach the OB where they undergo terminal differentiation (160).
In vivo tracking of NPC is possible with MRI and has been shown by Shapiro et al 2006 and others (144, 161, 161-163) in both rat and mouse models. Shapiro et al showed that NPCs are capable of endocytosing magnetic micron-sized iron particles (MPIO) and can be tracked in vivo along the RMS using MRI. These iron particles, coated with a fluorescent tag, are sensitive enough that only a few particles need to be engulfed by the NPCs (161, 164). The MPIOs are introduced to the NPC by injecting a bolus volume of the iron solution into the lateral ventricles. It is believed that a successful labeling of the NPCs is closely related to the injection site. The canula delivering the MPIO solution needs to be close enough to the SVZ so that the epithelial layer surrounding the NPCs will rupture and give access to Type A cells, which mainly are responsible for transporting the particles on the RMS (144). The fact that these migrating neuroblasts can internalize MRI contrast agents opens the possibility to label and track cells in situ and in vivo during longitudinal studies. Also, there is the potential to explore the different fates and migratory pathways of NPCs outside of the RMS and OB.

It has been shown that migratory neuroblasts have migrated laterly to the striatum and corpus callosum (CC) five days after an induced focal ischemia located in the frontoparietal cortex in adult rats, eventually expressing markers for oligodendrocytes. These cells did not demonstrate long term survival but the formation of neurons from NPCs in the OB as well as migration along the RMS appeared not to be disrupted (165, 166). Oligodenrocytes also have been observed when implanting NPCs from the SVZ in white matter tracks of the CC, showing the potential of NPCs for neuronal self-repair by myelin formation (167).

Similar migrations have been shown in critical cortical impact (CCI) by Salman et al. (168) After an ICV injection of a fluorescent dye, Salman et al. could follow the migration to the proximity of a contra lateral injury. Immunohistochemistry showed that these cells, primarily as astrocytes, contribute to the astroglial scar that is important for recovery of blood brain barrier and neuronal repair.

In addition to the endogenous labeling and tracking of NPC and their potential to institute neuronal tissue repair, other cell-based therapies are arising through the use of exogenous cell implants, such as the hMSCs. As described in Section 1.3.3, hMSCs have the ability to differentiate preferentially into mesenchymal phenotypes (41); however, they also can be transitioned along differentiation pathways that result in either neuronal or glial cells (44, 45). Due to their targeting and regenerative properties either by direct differentiation or supportive
chemokine production, hMSCs are a strong candidate for regenerative therapies, even in the case of neuronal damage resulting from either traumatic brain injury or ischemic stroke for which the vascular system is compromised. Additionally, as demonstrated in Chapter 2, hMSCs can be readily labeled and cultured with MRI contrast agents to allow for in vivo tracking following transplantation.

Common contrast agents, such as SPIOs, have been shown to easily be endocytosed by hSMCs (as described in chapter 2) without impacting viability, proliferation or differentiation (47). It also has been reported that intravenously injected hMSCs localize to the site of ischemic brain tissue (48) as well as in infarcted heart 2-3 days following tissue damage (49). In addition, MSCs secrete many stimulatory factors for regulating inflammation processes, as well as neuronal growth factors in cell cultures of damaged brain tissue extracts (48). Recent theories suggest that the primary function of hMSCs is not to provide replacements units of tissue but rather to create a beneficial microenvironment for the regeneration of tissue (50). It is without doubt that hMSCs have great possibilities to facilitate neuronal tissue repair. However, as with endogenously labeled NPCs, it is important to understand the migration and localization of hMSCs once implanted in vivo. The blood brain barrier (BBB) possesses a potential problem for implanted cells to access the parenchyma. However, when a CCI is applied, the BBB also will be disrupted; permitting hMSCs to home to regions of vascular disruption and even exit the circulatory system before being cleared by the kidneys.

The purpose of this chapter is to evaluate if endogenously and exogenously labeled cells that have been demonstrated migratory and therapeutic properties in association with a TBI can be tracked utilizing bimodal contrast agents with high field MRI.

4.2 Methods

This experimental setup consists of two independent studies, namely tracking of magnetically labeled NPC cells in association with a CCI resulting in a TBI and exogenous tracking of magnetically labeled hMSC transplanted by intra-arterial (IA) injection in conjunction with a TBI.
4.2.1 Animal models

Four week-old male Sprague-Dawley (SD) rats weighing approximately 150 g where used in both studies. All animal protocols were approved by the Florida State University Animal Care and Use Committee (ACUC). Prior to any surgical procedures animals were allowed to acclimatize one week after arrival to the research facility with food and water available ad libitum.

4.2.2 Cortical critical impact (CCI) and endogenous labeling of NPC

Animals were divided randomly into three groups: SHAM, with animals receiving TBI and ICV injection of PBS (N = 3); TBI-ICV, with animals receiving a TBI and injection of iron particles by ICV injection (N = 8); and ICV, with animals only receiving an ICV injection of iron particles without TBI (N = 3). Prior to surgery animals were anesthetized with isoflurane (The Bulter Company, Ocala, FL) and positioned in a stereotactic frame. The head of the rat was shaved and cleaned with iodine and 70% isopropanol prior to exposing the skull with a 1-1.5-cm rostral-caudal incision. The CCI procedure involved a 5-mm craniotomy immediately anterior to Bregma followed by a piston (diameter = 2.5 mm) impact induced at a speed of 2.0 m/s and 2-mm deep. Any bleeding was stopped before proceeding to the next step. The labeling of proliferating cells in the SVZ involved a 50-µL injection of a solution containing micron-sized (0.86-µm) iron oxides (MPIO) particles. These commercially available particles have an iron oxide core and are coated with crosslinked polystyrene/divinylbenzene as well tagged with a fluorescent dye (Bangs Laboratories, Fisher, IN). The particles are delivered suspended in surfactant and sodium azide, which was removed by centrifuging and re-suspending five times in sterile PBS to ensure that no toxins were present and to establish the correct neutral pH of 7.4 for injection into the ventricles or culture with implantable cells. The cleaned particles were injected 2-mm rostral, 2-mm lateral from Bregma and 3.5-mm deep at 12.5 µL/min with a 50-mL syringe using a syringe pump. The craniotomy normally exposed the ICV injection site and no additional burr hole for the cannula was needed. If a burr hole was required, an electrical drill was used to expose the parenchyma at the determined coordinates. After injection, the solution was allowed to equilibrate for one min following a slow removal of the cannula. After the surgery, the animal was sutured and placed on a heating blanket. As a result of decreased immobility after CCI and
to prevent excessive weight loss, food pellets were placed on the bedding inside the cage for easier access.

4.2.3 Exogenous labeling and intra-arterial injection of hSMC

Standard frozen human bone marrow-derived stem cells were obtained from the Tulane Center for Gene Therapy and were cultured following a method outlined in prior publications (77). Briefly, bone marrow aspirates from healthy donors ranging in age from 19 to 49 years were collected under an Institutional Review Board (IRB) approved protocol. Plastic adherent nucleated cells were separated from the aspirate, expanded on TCP petri dishes using α-MEM supplemented with 10% FBS at 37 °C and 5% CO₂, and cultured to passage five. All cells used in the experiments in this paper were seeded at passage six.

Prior to labeling with MRI contrast agent cells were seeded at 4,000 cells/cm² on plasma treated six-well plates (BD Falcon, Franklin Lakes, New Jersey) in 3 mL of culture media. Twenty-four hours after plating, the cells were washed with sterile PBS, and then 3 mL of fresh media was added followed by either SPIO (Feridex, Bayer Healthcare, Wayne, NJ; 11.2 mg/mL of Fe) or MPIO tagged with a fluorescent dye. With either type of particle, a mass of 56-µg Fe was added to each well and allowed to incubate for 12 h. After incubation, the SPIO/cell culture medium was removed; the cells were washed three times with sterile PBS, trypsinized and re-suspended in non-serum supplemented α-MEM. Immediately harvest, the cells were injected into an already surgically prepared animal.

Prior to harvesting the labeled cells, as described above, the surgical procedures begin with a CCI as described in Section 4.2.2. After any bleeding was stopped, the animal was sutured and placed in a supine position. Rats were transferred to a dorsal recumbent position, and the forelimbs were secured with tape. A gauze bolster was placed under the neck to clear the airway and facilitate isolation of the carotid artery with the tongue pulled to a lateral position to prevent airway occlusion. After shaving and sterilization with iodine and 70% isopropanol, a 2-3 cm midline incision was made ending no lower than the subclavicular line. Any bleeding was stopped. Blunt dissection was used to visualize the carotid artery behind the sternohyoid, omohyoid and sternomastoid muscles. Upon visualization of the carotid artery, it was gently separated from the vagus nerve. One silk suture was applied to the posterior region of the visible
carotid artery to occlude the vessel using a surgeon’s friction knot. Loose second and third sutures were applied to the anterior and medial regions of the exposed artery to secure the cannula. A small v-shaped incision was made in the artery between the posterior and medial sutures for the insertion of the cannula. The second and third sutures were tightened to secure the cannula. The already labeled and harvested cell solution containing approximately 2 million hMSC was transferred to a 1-mL syringe and injected slowly. The cannula was then removed and the second and third sutures were tightened completely to occlude the vessel permanently. In order to keep the hMSC from aggregating as well as to optimize cell viability, it was crucial to coordinate the surgical procedures, so that the CCI and the dissection of the carotid artery was done by the time the harvested hMSCs were ready to be implanted.

4.2.4. MR imaging

As indicated in Figure 22, animals were imaged 24 and 48 hours post surgery and then once every week to evaluate the injury and progression of migration. Animals receiving exogenous labeled hSMC were imaged at 24 and 48 h followed by two more scans the following weeks.

![Timeline of surgery and MR imaging](image)

**Figure 22:** Timeline of surgery and MR imaging

MR images were acquired on a 21.1-T (900-MHz) vertical magnet with a diameter of 105-mm and built entirely at the National High Magnetic Field Laboratory (NHMFL). The magnet was equipped with a Bruker Avance III spectrometer (Bruker Corp, Billerica, MA) and a
64-mm inner diameter high performance gradient (Resonance Research Inc, MA). All samples were acquired using ParaVision 5.1 software (Bruker Corp, Billerica, MA). A 35-mm homebuilt $^1$H birdcage coil tuned to 900 MHz was used to image all animals. The coil was equipped with a bite bar supplying oxygen and isoflurane anesthesia to the animal. A constant supply of 1.5-2% isoflurane was maintained throughout the imaging session. Respiration was monitored with a SA Instruments, Inc. (Stony Broke, NY) monitoring system. This system also supplied triggering for MR acquisitions in accordance with the respiratory cycle to reduce motion artifacts.

Image sequences were performed to evaluate the early progression and retrogression of the TBI, the formation a necrotic core and the migration of labeled cells. To image the neuronal injury, a multi-slice diffusion-weighted spin echo (DWSE) sequence was acquired in a coronal anatomical direction with two diffusion weightings, $b$ values = 0 and 1000 s/mm$^2$. The DWSE resolution was 200x200 µm with a slice thickness of 1 mm and TE/TR = 25/3750 ms. To visualize the edema associated with the TBI, a fat-suppressed, multi-slice $T_2$-weighted SE sequence was acquired in an axial direction with TE/TR=20/6000 ms, 100x100-µm resolution and slice thickness of 0.5 mm. To visualize the location of labeled cells, a 3D $T_2^*$-weighed gradient recalled echo (GRE) sequence was applied. This scan was acquired in a sagittal direction with 50x50x200 µm resolution and had a TE/TR=5/50 ms. Total image time was approximately 2 h/animal. After the last in vivo scan, the animals were perfused with saline and 4% paraformaldehyde, decapitated and scanned ex vivo with a high resolution (60x60x60µm) 3D GRE sequence with TE/TR = 5/100 ms.

### 4.2.5 Immunohistochemistry

The perfused brains were excised from the skull and placed in a 30% sucrose solution until they sank. Brains were cryosectioned to 25-µm thick slices using a sliding microtome (Thermo Scientific, Waltham, MA) and put into a cryo-protectant solution for storage at -20°C. Tissue sections were used for identify intracellular iron and fluorescent particles. In brief, tissue sections were washed six times in PBS. Sections were exposed to Prussian blue staining according (Sigma Aldrich, St Luis MO) to the manufactures instruction. Sections then were mounted on subbed glass slides, dried overnight and dehydrated using dilutions of ethanol following a clearing step with xylene.
4.2.6 Image processing

To segment regions of the brain with either TBI damage or labeled cells, 3D GRE scans were processed in Amira 5.3.3 (Visage Imaging, CA). Via manual segmentation guided by threshold limits, the hypointense areas corresponding to labeled cells within the RMS and OB or outside of the immediate vicinity of the TBI were chosen on a voxel-by-voxel basis while hyperintense areas corresponding to the TBI and its necrotic core were similarly selected. The segmented dataset was co-registered using the Amira affine registration tool. In this way, animals in the same group could be compared to other groups and time points.

Signal-to-noise ratio (SNR) measurements were done by summating three slices that covered the labeled RMS in the 3D GRE images. Regions of interests (ROIs) were placed consecutively from the start of the RMS going towards the OB. Additionally, ROIs were drawn in a noise region as well as tissue unaffected by iron. Figure 23 shows an example displaying the ROI placement for SNR calculation in a co-added image from a representative 3D dataset.

**Figure 23:** Summation of three slices from a 3D GRE image covering labeled cells on the RMS. Green boxes are examples on how ROIS are placed to calculate to calculate SNR.
4.3 Results

4.3.1 Cortical critical impact (CCI) and endogenous labeling of NPC

The aim of this study is to investigate if NPCs, which continuously migrate along the RMS and support the OB with neurons, will re-route to neurological damage, such as TBI. To this point, a limited number of animals have been analyzed due to either errant TBI or an unsuccessful labeling of NPCs. Of attempted surgeries, an overall success rates of 64% for the labeling of NPC cells was achieved as evaluated with T_2- and T_2*-weighted scans while the TBI was identifiable in 57% of CCI procedures as evaluated by DWSE images. For the SHAM group, two of the three animals received a successful TBI. For the ICV group, two of the three animals received a successful labeling of NPCs. Of the eight attempted TBI-ICV animals, four showed successful labeling with six displaying a successful TBI. In this group, only two animals received both a successful TBI and significant NPC labeling. The remaining animals received either a successful TBI and no ICV (N = 4) or a successful ICV and no TBI (N = 2), with the later two animals reassigned to the ICV group. Ultimately, the SHAM and TBI-ICV groups consisted of N=2 successful animals while the ICV group consisted of N=4 successful animals.

Figure 24 illustrates segmented images of labeled NPCs and their progression along the RMS during two of the five time points for representative samples from the ICV and ICV-TBI groups. To compare labeling within the same groups, segmented datasets where co-registered and fused using Amira. The combined datasets show two animals in the ICV group (Figure 24 a-c) while two animals from the TBI-ICV group are seen on the bottom row (Figure 24 d-e). The TBI is shown as a white area while the labeled cells are shown in light gray projecting from the SVZ toward the OB. Overlapping segmentations between the two representative samples within a group display an increased pixel intensity for the three (brain, TBI and NPC) segmented regions. The progression of labeled NPC along the RMS can be seen with possibly stronger labeling when a neuronal injury is present. At one week after surgeries, the RMS is fully labeled, and the NPCs continuously migrate towards the OB. In the OB, cells do not follow a specific path but randomly disperse from the termination of the RMS. This dispersion can be seen clearly with ex vivo images (Figure 25) for which higher resolution and signal can be acquired. In vivo, time limitations prevent higher resolution images to be acquired for the visualization of cells.
located in the OB. However, based on the *ex vivo* image (Figure 25a) at day 12 post surgery, it is apparent that labeled cells have reached the OB.

In Figure 25b, a 2D partition from a 3D GRE image at three weeks shows labeling of NPCs on the RMS (red ellipse). Around the perimeter of the TBI (yellow ellipse), hyperintensities can also be seen. This contrast is indicative of aggregated iron most likely contained within cells. Preliminary histological images, of 25-µm sections indicate both the fluorescent MPIO on the RMS as well as in the perimeter around the TBI as seen in Figure 26. Prussian blue images also reveal that the irons are present and aggregated inside cells on the RMS (Figure 26c). The fluorescent MPIO appear to be abundant and are seen in discrete spots corresponding to cellular locations, suggesting that they are intracellular in both the SVZ at the site of TBI. The sources of these cells are yet to be determined but they could either be from SVZ-originating NPCs that have homed to the neuronal injury or astrocytes that have phagocytosed free MPIOs.

**Figure 24:** Segmented and co-registered images of two animals in the ICV groups (a-c) and two animals from the TBI-ICV (d-e) group during three of the five time points.
Figure 25 a: Projection view of a segmented *ex vivo* 3D GRE data set at day 12 post injury of one animal receiving both TBI and ICV injection of iron particles. b: An *ex vivo* 3D GRE image at 3 weeks post surgery demonstrating the migration of labeled NPCs along and outside of the RMS. Labeled NPCs within the RMS are displayed within the red circle. The yellow circle show the TBI lesion with hypointensities on the perimeter indicating that iron oxides are present and most likely within cells.

Figure 26: Histology: a) Fluorescent particles aggregated in what appears to be inside cells at the site of injury, b) Florescent and aggregated particles on the RMS and c) Prussian blue staining of the RMS, revealing iron inside cells along the RMS.
As a control, Figure 27 illustrates an animal receiving an injection of PBS instead of iron particles and a TBI. Not surprisingly, no hypointensities can be seen in the ventricles or along the RMS.

Figure 27: 3D GRE image of a sham animal injected with PBS instead of iron particles in conjunction with a TBI. TE=5ms TR=50ms and 50x5x200 um resolution and b and c showing coronal images of diffusion a weighted scan over 1 week were the change in signal intensity changes form hyperintense to a signal void due to the necrotic core.

Figure 28 illustrates a series of images from DWSE scans that were utilized to confirm the presence of TBI. The images show the progression of the injury during the three week time period. The top row (Figure 28a) show images with no diffusion weighting (b = 0 s/mm²), effectively representing a T₂-weighted scan, while the bottom row (Figure 28b) displays diffusion weighting (b = 1000 s/mm²) over the progression of three weeks. At 24 and 48 hours post surgery, the injury consists of a penumbra where the water of the affected tissue has shifted from the extracellular space to the intracellular space. This shift restricts the diffusivity of water hence increasing the relative signal with diffusion weighting. By one week after the injury, the tissue closest to the impact has died, and a hypointensity signal loss is seen in the diffusion-weighted scans that correspond to the hyperintensity of the T₂-weighted scans. This region is now filled with bulk water, constituting the necrotic and unsalvageable core of the TBI.
To further quantify the effect of TBI on NPC migration, volume measurements and distances of traveled for NPCs along the RMS were performed with the segmented datasets, as presented in Figure 29. For an animal from the TBI-ICV group compared to the averaged values from the ICV group, the volume of labeled NPCs from segmentation appears to be larger for all time points. Using the first identifiable point at the initiation of the RMS as a fiduciary marker, the traveled distance by labeled cells within the RMS can be measured using the segmented 3D datasets. As with the volume measurements, the TBI-ICV representative sample displayed faster and more extensive migration compared to the averaged ICV data. These trends seem to indicate increased proliferation and migration of NPCs with an underlying TBI pathology, even along the normal pathway of the RMS. This increase could either be due to increase in cellular uptake of iron particles in the SVZ or increase in the number of migrating cells.

**Figure 28:** Diffusion-weighted images illustrating the progression of the TBI
Figure 29 a: Volume measurements of segmented labeled voxels on the RMS and OB and b: migrated distance from the ventricles toward the OB along the RMS. The average and standard deviation is available in the ICV group (N = 4), but due to limited surgical success for the TBI-ICV group, only a single representative sample is provided (N = 1).
In Figure 30, SNR measurements are presented for the ICV and TBI-ICV groups in Figure 30b. Interestingly for both groups at 24 h, all respective regions have about the same SNR value. At 48 h, the animals receiving a TBI show a drastic increase in SNR. By one week, this increased SNR in the TBI-ICV animal has returned to levels similar to the 24-h time point. After one week, SNR values in both groups are reduced progressively but the TBI-ICV animal displays more drastic decays in signal with time and final SNR values at the third week that is lower than the averages for the ICV animals. This drastic initial increase in signal is most likely due to water movement associated with TBI; however, the longer term decrease in signal is likely a result of the migration of labeled NPCs as they move along the RMS. Although the exchange of water between the intra- and extracellular environments cannot be decoupled from the SNR measurement, it is notable that the SNR for both groups decreases with time and that the ultimate SNR for the TBI-ICV sample is lower for each ROI than the in the ICV group.

4.3.2 Exogenous labeling and intra-arterial injection of hSMC

To assess the targeting of exogenously labeled and implanted cells in TBI, a preliminary study was performed to inject iron oxide-labeled hMSCs were via the carotid artery in animals immediately following TBI. Animals were imaged at 21.1 T at 48 h and 9 days as shown in Figure 31. To date, only one animal has received both a successful TBI and hMSC implant. The
TBI serve not only as a neuronal injury to which cell should migrate but also a means of disrupting the blood brain barrier (BBB), which otherwise would have limited cells from entering the parenchyma. At 48 h following TBI and implantation, SPIO-labeled hSMCs appear around the initial TBI site as dark spots seen in the inside the white circle in Figure 31. By day 9, this contrast has vanished indicating that cells have migrated away from the injury. Immunohistochemistry is under way to prove what types of cells are associated with the hypointense contrast.

![Figure 31: 3D GRE (TR= 20ms and TE=5ms) images at 48 h and 9 days post surgery of an animal injected with 1x10^6 hMSC pre-labeled with SPIOs. Insert show a diffusion weighted image illustrating the presence of TBI.](image)

### 4.4 Conclusion

#### 4.4.1 Cortical critical impact (CCI) and endogenous labeling of NPC

In this study, it was demonstrated that NPC cells from the SVZ continue to migrate and engulf iron particles in association with a TBI. In addition, cell migration and proliferation are potentially increased with evidence of re-routing to the perimeter of the TBI.

At this point, a limited number of animals have been analyzed mainly due to unsuccessful TBI or injection of contrast agents. The placement of the cannula and delivery of iron oxide nanoparticles into the ventricles has shown to be crucial. In order for the NPCs in the SVZ to
gain access to the particles, the bolus injection needs to penetrate the ependymal cell layer covering the SVZ. Unsuccessful cannula placements and subsequent injects did not locate the intended contrast agent consistently in close enough proximity to the NPC niche. Additionally, there was some inconsistency in the institution of the TBI. These difficulties limited the numbers of successfully prepared animals that were available for this assessment. However, over time, an overall success rate of 64% for labeling of NPC cells and 57% for TBI was achieved.

As shown in Figures 24 and 25, NPC labeling occurs even with the underlying presence of a neuronal injury. In fact, though statistical numbers are not available due to the low sample size, it appears that NPCs uptake of nanoparticles and migration not only continued but may have been enhanced because of TBI. In some cases, labeled NPC migration is evident as early as 24 and 48 h post surgery, but at one week post surgery, migration is seen for all animals regardless of their treatment group. The mechanism of particle uptake is unclear but is likely directly incorporated in undifferentiated NPCs in the SVZ and then transported along the RMS as the cells differentiate into neurons in the OB (161). When analyzing the migration speed and the volume of labeled cells in the RMS and OB, the TBI appears to stimulate the NPC labeling in the SVZ to either take up more particles or migrate faster compared to the ICV only group.

In the SVZ, there is a constant balance between cell death and proliferation. Research suggests that there might in fact not be an increase in cell proliferation in association with neuronal injury but rather a decrease in cellular death. Gotts and Chesselet (169) showed that bromodeoxyuridine (brdU)-labeled NPCs in conjunction with a distal focal ischemia induce a rapid increase in both cell death and proliferation seven days post injury. When the cell number increased, the two mechanisms became uncoupled, and an expansion of cells within the SVZ and RMS was seen (169). The increase in proliferation is seen in a wide variety of different types of ischemia (165, 169-172), which likely explains the increase in uptake of iron particles because particles are likely incorporated in the cells as they proliferate. The exact mechanism of activation of NPCs in the SVZ is unclear but it is believed that vascular events in ischemic animal models, such as increased vascular permeability, secretion of vascular endothelial growth factor (VEGF), and angiogenesis within the SVZ, may stimulate NPCs (173) With TBI having some commonality with respect to ischemia pathology, NPC expansion is most likely effected by similar vascular factors that promote proliferation, cell targeting and re-route NPCs to the injury.
Even though no direct evidence of migrating cells from the SVZ to the TBI is found from \textit{in vivo} MRI images, \textit{ex vivo} images (Figure 25b) of the lesion show hyperintense areas in the perimeter of the TBI. These large signal voids are created by aggregated iron, mostly inside cells as demonstrated by preliminary histology images. Images of the fluorescent particles suggest that MPIOs are located in the periphery of the injury (Figure 26). Cell specific antibody staining is in process and will determine the exact phenotype of these cells. In either case, it is likely that the MPIOs used to label NPCs are well distributed around the injury in cell types associated with NPC lineage. This finding is supported in a study by Salman \textit{et al.} suggesting that NPCs residing in the SVZ and labeled with fluorescent dye using ICV injection migrate to the most proximal areas of neuronal injury, participating in cellular remodeling and inducing an astroglia scar following TBI (168). In addition, for a stroke model, Gotts and Chesslet showed that brdU-labeled NPCs are present in the ischemic region but only express markers for astrocytes and oligodendrocytes without neuronal markers (165). Histological evaluation has been done by Sumner \textit{et al.} on the migrating iron labeled cells on the RMS (144). Results show that the phenotypes of these cells correspond to oligodendrocytes, astrocytes and neurons. With the introduction of an anti-mitotic agent in the SVZ, both precursor cells and MRI enhancement were eliminated. The present findings and these previous reports support the contention that MRI contrast is exclusively from NPCs leaving the SVZ and not from non-specific diffusion of the MPIOs.

The effect on cellular function after \textit{in situ} cell labeling is not fully known, but it is believed that the SVZ microenvironment may be altered by ICV injections as a result of inflammatory response. Not surprisingly, inflammatory cell activation has been seen with ICV injections of neuraminidase (174), but also with rhodamine microspheres (175) and even three weeks after injection of 1.63-\(\mu\)m diameter polystyrene/divinylbeneze fluorescent iron particles (163). These inflammatory processes can affect adult neurogenesis (176). No inflammatory response was evident in this study, potentially because the smaller particles (0.86 \(\mu\)m) used in this study might not be as disruptive to the ependymal cell layer. Therefore, the smaller particles may have induced less inflammatory response in the SVZ at the expense of reduced labeling and transfection efficiency. These smaller particles also will impact the generated MRI contrast by inducing different patterns of susceptibility perturbation for labeled cells. Compared to other studies using a larger 1.63-\(\mu\)m iron particle, contrast in the current study appears very similar.
(144, 161); however, previous studies have utilized much lower magnetic fields (>11.75 T) than the 21.1-T magnet, which will provide more susceptibility contrast for a reduced amount of SPIO, as seen in Chapter 2.

The endogenous labeling and tracking of NPCs with iron based contrast agents has been shown previously (144, 161, 163, 164). To date, these techniques have been limited in their application to neurodegenerative diseases, with no MRI-based studies evaluating the adult brain. The current study implements a neuronal injury in which these migrating cells have shown by *ex vivo* processing of tissue (167, 169) to potentially be involved in neuronal repair or salvage operations. This study shows that it is possible to label NPCs with an ICV injection with bimodal nanoparticles in conjunction with a TBI. The TBI appears to stimulate proliferation, resulting in increased contrast seen along the RMS and OB. Histological images verify fluorescent particles at the site of the injury likely migrating from either the SVZ or leaving the RMS to home to the site of injury.

### 4.4.2 Exogenous labeling and intra-arterial injection of hSMCs

The direct intra-arterial injection of SPIO-labeled hMSCs through the carotid artery has been demonstrated as a potential route for stem cell treatment for neuronal injury. The direct route to the brain minimizes loss of cells otherwise seen with intraventricular injection (IV). MRI images suggest that hMSCs are only present for a short amount of time and gone after a week. Histology is underway to identify the cell phenotype that is associated with this hypointense contrast. This technique shows potential for future work involving cellular therapies in involving neuronal injury.
CHAPTER FIVE

CONCLUSIONS

This dissertation investigates the usage of contrast agents at ultra high magnetic fields (>11.75 T) with a special focus on intracellular contrast agents. The focus has been on describing the efficacy of readily and commercially available iron oxide agents to novel high field agents based on quantum dots and dysprosium for cellular labeling as well as in vivo cellular tracking with a micron size, bimodal iron oxide particles.

The findings suggest that intracellular iron oxides (SPIO) imaged in a tissue mimicking phantom has limited benefits at 21.1 T compared to 11.75 T. In addition, this same study shows that the microglial cell line used, once activated with LPS, can readily engulf nanometer sized particles. Efforts made with the same contrast agent also show that labeling of hSMC are possible with the same type of particle without any effect on viability and proliferation but with a decreased ability to be detected in long term tracking due to dilution of intracellular particles as the cells proliferate. Interestingly, preliminary results with these cells cultured in hypoxic atmosphere, being their natural microenvironment, show increased cytotoxicity after 24 hrs of iron exposure. These results are potentially important when implanting these types of cells in ischemic animal models.

Chapter 4 describes the utilization of another type of iron-based contrast agent, namely a bimodal fluorescent micron seized iron oxide particle. This particle was used for endogenous labeling of neuroprogenitor cells in conjunction with a neuronal injury, in this case a critical cortical impact creating a TBI. The results show that cells located in the SVZ will, despite the injury, continue migrating along the well defined RMS. Results indicate an increased proliferation due to the brain injury as seen by the increased speed and volume labeled cells. In addition, ex vivo images suggest that differentiated NPC have migrated from the SVZ to the perimeter of the brain injury carrying the fluorescent iron particles. One of the major limitations in this work is the usage of the large volume of the relatively big iron particles that very efficiently destroy any signal from tissue in close proximity of the particles. These “blooming” artifacts are especially prominent around the ventricles where the particles are injected. With the
development of a novel contrast agent for high magnetic fields, described in Chapter 3, we have shown a dysprosium based MRI contrast agent utilizing a fluorescent quantum dot for bimodality. This agent provides $T_2$ contrast once internalized inside cells and immobilized in a tissue mimicking phantoms, with potential for desired $T_1$ contrast. This contrast agent does not create distortions that are detrimental in the manner that iron based particles destroy information in the vicinity of the agent. With future usage of this particle, in vivo imaging and cell tracking studies like those of Chapter 4 should be enhanced. Additionally, the available contrast from the Dy-QD will provide means of: a) displaying positive to negative contrast changes as NPCs are transfected from the bulk cerebrospinal fluid of the ventricles, b) providing a platform for improving cell transfection using particles functionalized with cell penetrating peptides, and c) modifying the genetic makeup of NPCs to induce or increase chemokine secretions to aid in regenerative or protective processes. These efforts are currently underway.

Using such agents, potential information also could be gathered on migrating cells along normal pathways (such as the RMS) and re-routed pathways to brain injury. In addition, this particle has room for improvement to increase the payload of Dy$^{3+}$ and also for modifications with cell penetrating peptides, as has been showed in Chapter 2. Modifications also could include peptides and antibodies for a more specific targeting of cells. Antibody targeting for more specific cell phenotypes in the SVZ would require fewer particles, hence reducing the potential impact on surrounding tissue. Together with the potential for generating $T_1$ contrast utilizing either dysprosium or gadolinium, these modifications would permit labeled cells to be more easily identified and classified.

This dissertation shows that therapeutic relevant exogenous cell lines such as neuronal microglia and hSMCs can be labeled and detected at high magnetic fields but with limited benefit when using existing SPIO particles. These cell lines have the potential to be implanted in association with neuronal injuries to either promote healing or provide timelines for disease progression that may be critical to process-specific therapeutic interventions. The therapeutic properties of these cells differ. While microglia cells are first responders to an injury, cleaning debris and secreting anti-inflammatory factors, hMSCs are more involved in the repair and restoration of neuronal tissue. The benefit of hSMCs likely is not by differentiating to neurons but rather creating either a suitable microenvironment for tissue formation or serving as a molecular beacon to endogenous cell line. For example, there is distinct possibility that NPCs
may be stimulated by or attracted to hSMC secrections to form new neural cells at the site of injury. The work, nanoparticles and high field techniques developed as part of this dissertation will facilitate the timeline of these events to determine when in the neurodegenerative process these cells are present and active at a neuronal injury. Future work that incorporates more sensors, such as paramagnetic chemical exchange saturation transfer (PARACEST) agents, on high field optimized contrast agents would extend the functionality of these particles to enable multiple cell line tracking and the monitoring of intracellular parameters such as pH, oxygen tension and temperature.
APPENDIX A

IACUC DOCUMENTATION

MEMORANDUM

TO: Dr. Cathy Levenson
    Department of Biomedical Sciences
    College of Medicine

FROM: Dr. Elaine M. Hull, Chair
      Animal Care and Use Committee

SUBJECT: Protocol #0916

DATE: June 10, 2009

"YOUR NEW PROTOCOL IS APPROVED"

The Animal Care and Use Committee approved new Protocol #0916, "Nutrient-Drug Interactions: Nutritional and Pharmacological Treatment of Traumatic Brain Injury", for proposed vertebrate animal use at the May 28, 2009 ACCC meeting. You are approved for the following species and numbers for the proposed protocol approval period.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number Animals Approved</th>
<th>Protocol Approval Expiration Date</th>
<th>Rewrite Due</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>200</td>
<td>May 31, 2012</td>
<td>April 1, 2012</td>
</tr>
</tbody>
</table>

Enclosed for your records are:

✓ A copy of the Committee Comments
✓ A copy of the Protocol
✓ The original of page one for signature and return in the enclosed envelope

When you order animals on this protocol, please remember to convey the ACTC number to the IAR at 644-4262. In addition, if you do not currently have animal housing or procedural space assigned or should you need additional animal housing or procedural space, please make a request for space in writing to the Biomedical Advisory Committee (BAC) care of Kristin Aule at kauel@fsu.edu. Animals will not be ordered unless adequate animal housing/procedural space is confirmed by the IAR Facility Manager.

We appreciate your contribution to assuring that animal research at Florida State University complies with federal guidelines and regulations. Let us know if we can be of further assistance.

EMH/C
Enclosures
MEMORANDUM

TO: Dr. Yung Ma
   Dr. Samuel Grant
   Department of Chemical and Biomedical Engineering
   College of Engineering

FROM: Dr. Paul Trombley, Chair
   Animal Care and Use Committee

SUBJECT: Protocol #1036

DATE: November 18, 2010

"YOUR NEW PROTOCOL HAS BEEN CONFIRMED AS APPROVED"

The Animal Care and Use Committee confirmed approval of your new Protocol #1036, "Transplantation of Culture Expansional hMSC in Stroke Treatment", at the October 27, 2010 ACUC meeting. This approval was originally approved through the Designated Member Review process on October 4, 2010. You are approved for the following species and numbers for the proposed protocol approval period:

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<th>Protocol Approval Expiration Date</th>
<th>Requiring Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague Dawley (SD) Rat</td>
<td>100</td>
<td>October 4, 2013</td>
<td>September 1, 2013</td>
</tr>
</tbody>
</table>

Enclosed for your records are:
- The original Committee Comments
- A copy of the Protocol
- The original of page one for signature and return in the enclosed envelope

When you order animals on this protocol, please remember to convey the ACUC number to the LAR at 644-262. In addition, if you do not currently have animal housing or procedural space assigned or should you need additional animal housing or procedural space, please make a request for space in writing to the Biomedical Advisory Committee (BAC) care of Kristin Antcz
at icspecifeml.edu. Animals will not be ordered unless adequate animal housing/procedural space is confirmed by the LAR Facility Manager.

We appreciate your contribution to assuring that animal research at Florida State University complies with federal guidelines and regulations. Let us know if we can be of further assistance.

PQT/ki]
Enclosures
APPENDIX B

DISsertation Presentation

Intracellular MRI Contrast Agents for High Magnetic Fields

Jens Rosenberg
Florida State University
FAMU-FSU College of Engineering
Department of Chemical & Biomedical Engineering

Outline

- General Background
- Intracellular SPIO at high magnetic fields
  - Effect of magnetic field on intracellular localization
- Intracellular bimodal contrast agent
  - Effect of magnetic field on intracellular localization
- In vivo tracking of progenitor cells
  - Effect of magnetic field on intracellular localization
- Conclusions

MRI Contrast by T_2 & T_1

T_2 = Echo Time
T_1 = Rep. Time

Signal

CSF
White/Gray Matter

\[ S = S_0 e^{-\frac{TR}{T_1}} \]

\[ S = S_0 e^{-\frac{TE}{T_2}} \]
Outline

- General Background
- Intracellular SPIO at high magnetic fields
  - Aim 1: In vitro cellular transfection and intracellular localization
  - Intracellular bimodal contrast agent
    - Aims: Localization and transfection of nanoparticles
  - In vivo tracking of neuroprogenitor cells
- Conclusions

Background: MRI contrast agents

- $T_1$/$T_2^*$ Contrast
  - Great sensitivity
  - $T_2^* = \gamma B_0 M_r / T_2$ (relaxivity)
  - Well-known chemistry
  - Can be functionalized for cell-specific targeting

\[
S = S_0 \left( 1 - \frac{1}{T_1} - \frac{1}{T_2} \right)
\]

Background: Bv2 cells

Microglia cells (rat Bv2) ⇒ neuronal macrophages

- Amoeboid – Total development
- Ramified – Most abundant microglia in CNS
- May present multipotent stem cell characteristics

- Activated – Invasion of viruses, bacteria, etc.
  - Secrete anti-inflammatory mediators
  - Linked to Alzheimer’s disease, amyotrophic lateral sclerosis and potentially other neurodegeneratives by chronic activation.

Background: Limitations of SPIO

- Loss of signal creates “black hole”
- Susceptibility effects distort tissue ⇒ “Blooming” artifact
- Signal voids can be mistaken for image artifacts, blood vessels or other structures
  - Particularly pronounced at higher magnetic fields due to increased local field inhomogeneities

Methods: Cell culture, labeling & immobilization

- Bv2 Microglia Cells
  - Inoculate cell-seeded culture dishes
  - DMEM supplemented with non-essential amino acids
  - Incubation at 37°C and 5% CO$_2$
- SPIO transfection of Bv2 cells
  - 24 hrs prior to incubation, cells were activated with LPS (10mg/mL)
  - SPIOs were allowed to incubate for 6 hr before harvesting
  - Cells were washed three times with PBS and then trypsinized
- Tissue phantom for MRI: Cell immobilization
  - Labeled and unlabeled cells were immobilized in 3% low-temperature agarose in defined layers for simultaneous imaging and analysis.
Objective: Investigate effectiveness of SPIOs at high magnetic fields and evaluate the uptake properties of SPIO with Bv2 cells.

MRI parameters: 11.75 and 21.1 T

**Relaxation measurements:**
- $T_1$, $T_2$, & $T_2^*$

**High Resolution Images:**
- 3D GREs: TE/TR=5/100ms, Res.=50-μm isotropic

Methods: Tissue phantom & high field MRI

- **SPIO dosing:**
  - 11.75 T & 21.1 T
  - High Resolution Images: 3D GREs TE/TR=5/100ms, Res.=50-μm isotropic

Results: Intracellular SPIO contrast between fields

- **Cell Dosing:**
  - 11.75T vs 21.1T
    - No apparent difference between field strengths

- **Results:**
  - 11.75 T: $m=1.3043\times10^{-4}$ s$^{-1}$/cells, $R^2=0.98$
  - 21.1 T: $m=9.3687\times10^{-5}$ s$^{-1}$/cells, $R^2=0.70$

* Sig. to 200K cell layer at 21.1 T (Tukey post hoc $p<0.05$)
Conclusions: SPIOs at high magnetic fields

- SPIOs had no impact on BV-2 cell viability (>95%)
- Compared the two fields, no overall difference in $R_1$ or $R_2$
- $R_1$ is sensitive to Fe$^{3+}$ cell count, but no field enhanced detection
- $T_2^*$ contrast shows significant differences between fields
- Increased detectability to susceptibility differences with higher fields
- SPIOs display only limited benefits between these two high field strengths

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Background: SPIO-labeled hMSCs

- Human Mesenchymal Stem Cells (hMSC)
  - Human regenerative properties, including within the nervous system
  - Implanted cells have been localized to ischemic brain tissue
  - Pluripotent stem cells
  - Differentiate into tissue-specific cells (osteocytes, chondrocytes, adipocytes)
  - Recent theories suggest the prime function is to create a beneficial microenvironment rather than tissue replacement

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Manuscript in review

Conclusion:

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  - Implanted cells have been localized to ischemic brain tissue
  - Pluripotent stem cells
  - Differentiate into tissue-specific cells (osteocytes, chondrocytes, adipocytes)
  - Recent theories suggest the prime function is to create a beneficial microenvironment rather than tissue replacement

Methods: Cell culture, labeling & immobilization

- hMSCs
  - Maintained with standard culture methods
  - α-MEM supplemented with non-essential amino acids
  - Incubation at 37°C and 5% CO$_2$
- SPIO transfection
  - Cells were plated 24 hrs prior to SPIO incubation
  - SPIOs added in different concentrations to separate wells and incubated for 6 h
  - Cell media was removed, cells were rinsed 3x with PBS, and fresh media was added
  - Cells were cultured for 1, 7, or 14 days
- Tissue phantom for MRI: Cell immobilization
  - Labeled and unlabeled cells were immobilized in 1% low temperature agarose in defined layers for simultaneous imaging and analysis

Results: Initial SPIO labeling & culture

- SPIOs had no impact on Bv2 cell viability (>95%)
- Comparing the two fields, no overall difference in $R_1$ or $R_2$
- $R_1$ cannot distinguish between increased cell or SPIO loading
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Results: Prussian Blue staining during culture

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- $T_2^*$ contrast shows significant differences between fields
- Increased detectability to susceptibility differences with higher fields
- SPIOs display only limited benefits between these two high field strengths
Results: SPIO-labeled hMSC cell assays

No significance was evident for:
- Cell growth
- Glucose consumption
- MTT
- RT-PCR
- HIF 1a, 2a
- ALP
- Osteolin
- Oct4
- Rex1
- RunX2
- Differentiation
- ALP, Alizarin Red
- CFU-F

Results: SPIO-labeled hMSCs under hypoxia

• SPIO-labeled hMSCs experience more cytotoxic effects under hypoxia
• Contrary to a previous study of non-SPIO labeled cells for which hypoxia enhanced proliferation & tissue formation

Background: Dysprosium

Dy³⁺ have improved relaxation with higher field strength

\[ R_{1,2} = R_{1,2}^{\text{Si}} + R_{1,2}^{\text{Dy}} \]

Background: Quantum Dots (QD)

- Excellent for cell labeling
  - Transfection
  - Surface functionalized with peptides for targeting

Conclusions: SPIO & hMSCs at high magnetic field

- SPIO can be readily engulfed by hMSCs w/o transfection.
- Internalized SPIO is sufficient for MRI contrast with dilution seen over time, with higher loadings preferable.
- No effect on viability, colony formation, differentiation.
- Hypoxic environments and SPIOs have negative effects on viability.

Conclusions: SPIO labeling of stem cells is possible & may not impact viability or expression...
... but long term MRI tracking requirements and cytotoxicity of challenged cells may be problematic

Outline

- General Background
- Intracellular SPIO at high magnetic fields
- Intracellular bimodal contrast agent
- In vivo tracking of neuroprogenitor cells
- Conclusions

• Chemical Formula: Dysprosium

• Backdrop: Quantum Dots (QD)
### Bimodal contrast agent:

**Objective:** Development & analysis of an intracellular bimodal high field contrast agent based on Dy and QD

**Methods:** Cell culture, labeling & immobilization
- Chinese Hamster Ovarian (CHO) Cells
  - Maintained with standard cell culture methods
  - DMEM supplemented with non-essential amino acids
  - Incubation at 37°C and 5% CO2
- CHO cell transfection with Lipofectamine2000
  - Transfection done 24 hours before transfection of cells at 90% confluency
- Samples were allowed to incubate for 24 hours
- Cell setup for MRI
  - Cells immobilized in agarose
  - ~150,000 cells immobilized in 1% agarose

**MRI methods:** Bimodal contrast agent

**2T 1.5 T MRI Magnet**
- Bruker Avance Spectrometer and Paravision
- UWB Magnet equipped with Bruker Avance Spectrometer and ParaVision
- 1H Birdcage coil, resonating at 900 MHz
- Spin Echo and Gradient Recalled Echo sequences
- Resolution = 148x80x1000 μm
- Matrix: 128x128
- BW: 75 kHz

**Relaxation measurements:**
- T1, T2, & T*2 measured
- High-resolution images: 3D GREs

**Results:** QD-Dy in agarose solution

**Quadratic regression yields:**
\[ R_2(s^{-1}) = 5.74 + 57.4[Dy] - 9.60[Dy]^2 \]
(R2 value = 0.9951)

Increasing Dy3+ concentration (with increasing QD mass) demonstrate increasing R2 relaxation

**Results:** Cell viability

Flow cytometry viability data based on AnnexinV and Alexa Flour dye

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>QD-PEPTIDE 4</th>
<th>QD-CAAKA-DOTA-Dy 5</th>
<th>QD-CAAKA-DOTA 4</th>
<th>Unlabeled CHO cells 5</th>
<th>Agarose 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (ms)</td>
<td>3094.8</td>
<td>3022.7</td>
<td>3020.0</td>
<td>3190.4</td>
<td>3453.8</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>304.2</td>
<td>52.3</td>
<td>40.7</td>
<td>60.9</td>
<td>245.8</td>
</tr>
<tr>
<td>Number of Samples</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>a</td>
</tr>
</tbody>
</table>

**Significantly different from T<sub>1</sub> of unlabeled CHO cells as assessed by Tukey’s HSD (p < 0.07)

**Significantly different from T<sub>2</sub> of QD-CAAKA-DOTA-Dy as assessed by Tukey’s HSD (p < 0.07)

**Results:** MRI and cell imaging

Evaluation of the bimodal agent in CHO cells
Results: Fluorescent imaging

![Fluorescent images showing the localization of QD-CAAKATat-DOTA-Dy within CHO cells.](image1)

![Ultra-high field MRI images showing T1W and T2W signal changes.](image2)

Discussion: Bimodal contrast agent

- **Comparison to other particles:**
  - QDs favorably to other particles: $T_1$, $T_2$

<table>
<thead>
<tr>
<th>Agent</th>
<th>$T_1$ (ms)</th>
<th>$T_2$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QD-CAAKATat-DOTA-Dy</td>
<td>2977.9</td>
<td>55.5</td>
</tr>
<tr>
<td>Lipofectamine &amp; CHO cells</td>
<td>3088.0</td>
<td>100.5</td>
</tr>
<tr>
<td>Agarose</td>
<td>3051.0</td>
<td>100.9</td>
</tr>
</tbody>
</table>

- **Modifications:**
  - Self-transferring with Tat peptide
  - Lower $T_1$, longer peptide – more water access
  - Other modifications to improve contrast
  - Branched peptides to increase lanthanide delivery

- **Potential usage for:**
  - In vivo cell migration
  - Monitor cells longitudinally with MRI and fluorescence

Background: Endogenous cell labeling

- **Neuroprogenitor (NP) cells with MPIOs and tracked on RMS: Shapiro et al Neuroimage 2006**
- **NP cells found in the subventricular zone (SVZ) of lateral ventricles**
- **Migrate along rostral migratory stream (RMS), differentiate, and form granule cell neurons in the olfactory bulb (OB)**
- **Can potentially migrate to neuronal damage (stroke, TBI)**

**Objectives:**
- Track NP cells on RMS & possible re-routing in association neuronal injury

Summary: Bimodal contrast agent

- Successful development of bimodal high field agent
- The nanoparticle agent internalized into CHO cells showed $T_1$, $T_2$, contrast and detectability in tissue mimicking phantom
- The quantum dot fluorescence revealed its intracellular localization within peri-nuclear endosomes
- Incorporation of Tat peptide enhanced the particle to be self transferred

**QDs provide a platform for enhanced delivery & contrast with field-sensitive MRI contrast agents as well as multi-modality imaging & functionalization potential.**

Manuscript published: Rosenberg et al MRM 64 (3) 2010

Outline

- **General Background**
- **Intracellular SPIO at high magnetic fields**
- **In vivo tracking of neuroprogenitor cells**
- **Conclusions**
**Background: Neuroprogenitor (NP) Cells**

- Type E: Ependymal cells
- Type A: Migrating neuroblasts
- Type C: Actively proliferating cells scattered in the SVZ
- Type B: Tube-like cells with astrocyte characteristics

**Background: Traumatic Brain Injury (TBI)**

- TBI is a two-stage injury:
  - Mechanical impact forces that cause distortions and destruction
  - Biomolecular and physiological response
    - Hypoxia/ischemia, edema, intracranial pressure and associated vasculature changes
    - Glutamate release
- Inflammatory response:
  - Neutrophils, activated microglia, astrocytes and neurons
  - Microglia have been shown to be present as early as 4 hr post injury and play an important role in tissue damage/repair.

**Methods: Surgery**

- **TBI:**
  - Animals anesthetized and put in a stereotactic frame
  - Craniotomy rostral to Bregma
  - Cortical Critical Injury (CCI)
    - 2 mm deep
    - Impact velocity: 2.2 m/s
  - Intra-cerebral Ventricular (ICV) injection:
    - 0.86 µm in diameter iron oxide particle tagged with fluorescent red dye (680 nm)
    - 50 µL injected at RC+2, LR +2, DV -3.5 mm from Bregma

**Methods: MRI**

- **21.1-T UWB vertical magnet**
  - Equipped with a Bruker Mri® 75 gradients and Bruker III spectrometer
  - 33-mm RF birdcage coil
  - Rodent probe equipped with:
    - Bite bar for support and oxygen/anesthesia
    - Respiratory monitoring & acquisition gating
  - MRI data is processed in PV5.1 and Amira 5.3.3

**Methods: MRI**

- **T2W RARE, axial orientation**
  - TE/TR= 20/6000 ms, 50 µm in plane resolution, 0.5 mm slice thickness
  - Fat suppression, NEX=2 and triggering used

- **3D GRE, sagittal orientation**
  - TE/TR= 5/50 ms, 50x50x200 µm resolution, 40 min
  - No fat suppression, NEX=1 and triggering used

- **Diffusion Weighted SE, coronal orientation**
  - TE/TR= 25/3750 ms, 2 b values (0 and 1000 s/mm²)
  - 200 µm resolution, 40 min
  - No fat suppression and triggered per slice

  - Total scan time ~ 2h/animal

**Methods: Study outline**

- **Animal groups**
  - TBI-SHAM: TBI and ICV of 1xPBS (N=2)
  - TBI-ICV: TBI & ICV injection of MPIOs (Bangs-Red) (N=2)
  - ICV: Injection of same MPIO as above without TBI (N=4)

- **Overall success rate:** TBI 57% and ICV 64%

**Surgery and MRI timeline:**

1. Surgery
2. 24 h
3. 1st MRI
4. 48 h
5. 2nd MRI
6. 1 Week
7. 3rd MRI
8. 2 Weeks
9. 4th MRI
10. 3 Weeks
11. 5th MRI
12. Sacrificing of animals followed by ex vivo scans
Results: In vivo imaging (TBI-SHAM)

Development of the TBI as imaged by DWI

Results: In vivo cell tracking (TBI-ICV)

Development of the TBI as imaged by DWI and 3D GRE

Results: Ex vivo cell tracking (TBI-ICV)

Results: Cell tracking by 3D segmentation

Results: Cell volume & Migration distance

Results: Ex Vivo cell tracking (TBI-ICV)

Signal-to-Noise Ratio (SNR):
- Summation of slices covering the RMS
- Regions of Interests (ROIs) placed to cover RMS & OB

\[ SNR = \frac{S - S_{\text{noise}}}{\sigma_{\text{noise}}} \]
**Results: In vivo quantification of cell tracking**

SNR measurements

- Increase SNR at 48 hrs for the TBI-ICV group
- No increase in SNR for the ICV group

**Results: Immunohistochemistry (IHC)**

**Discussion: Endogenous cell labeling**

- Previously: Endogenous labeling of NP Cells
  (Shapiro et al. 2006 Neuroimage, Sumner et al. 2009 Neuroimage, Vreys et al. 2010 Neuroimage)
- Contrast is only due to labeled cells and not to non-specific MPIO diffusion
- Proliferation may not be increased
  - Constant cell proliferation and cell death in SVZ
- NP cells can migrate to TBI → astroglial scar
- Smaller particles (<0.86 µm) affect relative contrast
  - High field potentially can compensate and reduce internal Fe +3 needs

**Conclusions: Endogenous cell labeling**

- Labeled NP cells can be tracked in concert with TBI
- Visualized and quantified cell number expansion along RMS associated with TBI → SNR provided an in vivo marker of time-sensitive migration
- In vivo MRI and IHC showed MPIO-GFAP + cells at the site of injury with some (though less) MPIO-NeuN + cells
- Artifacts from Fe +3 induced "blooming" of ventricles limits tracking of NP cells close to ventricles and SVZ
- Sample size is a limiting factor but more experiments are planned and the trends are very encouraging

**Overall Discussion and Summary**

- SPIOs: Limited benefits at high magnetic fields
- Microglia and hMSCs
- Nanoparticle uptake for MRI tracking
- Diagnostic and therapeutic properties are beneficial for cell therapies aimed at longitudinal studies of neurodegeneration
- Bimodal high-field MRI contrast agent based on QD and Dy
- Modifications to improve contrast, function, and cell targeting
- Significant high field (<1 T) improvements & potential
- In vivo high-field cell tracking
- Endogenous labeling of NP cells in the presence of a TBI or other injury
- MRI visualization of cell expansion on RMS and re-routed NP to injury

**Ongoing Work: TBI with SPIO-labeled hMSCs**

- TBI followed by IA injection of 1 million hMSCs
- hMSC culture and implantation
  - Cells seeded into 6-well plates (~1 million cells / plate)
  - Incubated for 12 hr with CA (Feridex and one try with Bangs Green)
  - Trypsinized and re-suspended in 1 mL of αMEM.
  - Injected slowly into the carotid artery
- In vivo MRI following IA injection: 24 hr, 48 hr & 9 days
Results: hMSC transplantation for TBI

MRI appears to reveal labeled hMSCs in or around TBI
+ Clearance of contrast within 9 days may indicate:
  → "removal" of hMSCs from the injury site
  → Proliferation or transformation

Future work:
- Endogenous NP cell transfecting using QD-Dy particles
  → $T_1$ in ventricles shifts to $T_2$ in NP cells
- QD-Dy modifications:
  → Improved nuclear $T_1$ contrast
  → Cell specific targeting and transfection of cells in the SVZ
  → what type of cells in the SVZ is migrating to injury, Type A or B
- Genetic manipulation
  → Expression of fluorophores with iron oxide SPIO labeling
  → Evaluating different lanthanoids such as Europium, Thallium
  → PARACEST agent to assess intracellular environment in vivo
  → pH, intraocular oxygen tension, temperature
- Multi-tracking: Tracking of two or more relevant cell lines
- Utilizing microglia and hMSC as biomarkers and therapies for acute and chronic degenerative diseases

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- Dr. Strouse, Dr. Kogot, and Chris Ridel – FSU Chem
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- Dr. Grant and fellow lab members
REFERENCES


41. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine J Cell Physiol 2007.


BIOGRAPHICAL SKETCH

Jens T. Rosenberg

EDUCATION

PhD in Biomedical Engineering
The Florida State University, College of Engineering
The National High Magnetic Field Laboratory
Florida State University, Tallahassee FL
GPA: 3.7/4.0

Course work in Business economics and Effective communication in English 2006
Stockholm University, Stockholm, Sweden

Master of Science in Engineering, Materials Technology October 2005
Royal Institute of Technology, Stockholm, Sweden
GPA: 3.8/5.0
- Master thesis in biomedical engineering for master degree Spring 2005
  University of Florida, Gainesville, Florida
- Course work in biomedicine for master degree 2004
  Karolinska Institute, Stockholm, Sweden
- Management and Statistics course work for master degree Spring 2003
  University of Adelaide, Adelaide, Australia

RESEARCH EXPERIENCE

Animal Surgery
- Intra cerebral ventricular injection (ICV)
  Injections of MRI contrast agents for endogenous tracking of neural progenitor cells
- Intra arterial injections of labeled stem cells
  Arterial injections of human mesenchymal stem cells labeled with MRI contrast agents
- Traumatic brain injury (TBI)
  Induced TBI by critical cortical impact to track cellular response of endogenous
  and exogenous labeled cells
- Perfusion
  Fixation of tissue for ex vivo imaging and histology

Magnetic Resonance Imaging
- Magnet systems
  21.1 T Ultra Wide Bore magnet equipped with Bruker avance III spectrometer and Para Vison 5.1
  and an 11.75 T magnet system equipped with Bruker Avance console and micro 2.5 gradients.
  - Relaxometry
    $T_1$, $T_2$ and $T_2^*$ weighted high field MRI sequences to classify novel high field and
    intracellular contrast agents
- Paramagnetic Chemical Exchange Saturation Transfer (PARACEST)
Evaluation of PARACEST agents at high magnetic fields

- **In Vivo imaging of animal models**
  Imaging of rats and hamster animal models using, 3D, T2 weighted and diffusion weighted high field imaging sequences

- **Ex vivo imaging**
  Imaging of excised central nervous system using 3D high resolution sequences and diffusion tensor imaging

**Cell Culture**

- Culture of human and non-human cell lines for treatment with MRI contrast agents

**PROFESSIONAL EXPERIENCE**

**Teaching Assistant**

- Mass and Energy Balance 2010
- Quantitative Anatomy and Physiology II 2009
- Quantitative Anatomy and Physiology I 2009
- Thermodynamics 2008
- Measurement and Transport Phenomena Laboratory 2007
- Chemical & Biomedical Engineering, Thermodynamics 2006

**Substitute Teacher**

- High School and middle school teacher for Mathematics, Chemistry, and Physics, 2004-2006

**TRAINING**

- **Bio Safety Level II** 2007
  FSU Institute of Molecular Biology
- **Magnet Handling** 2006
  Training on 900 MHz magnet at the NHMFL, Tallahassee
- **Animal Care and Use** 2006
  FSU Laboratory Animal Resources
- **PIE Teaching Certificate** 2006
  Program for Instructional Excellence, FSU

**PROFESSIONAL ACTIVITIES**

- Golden Key Honor Society, Member 2009-present
- Biomedical Engineering Society, Member 2008
- International Society of Magnetic Resonance in Medicine (ISMRM) 2007-2011
  Joint annual meeting Berlin, Toronto, Honolulu, Stockholm, Montreal
- Gordon Research Conferences: In vivo Magnetic Resonance, Poster presentation 2008
- Experimental Nuclear Magnetic Conference, Poster presentation 2007
- Company Host, Stockholm University, Sweden Department of Business 2006
- Material Science Student Union, Royal Institute of Technology, Sweden 2004

**COURSE PRESENTATIONS**

- **Graduate Student Seminar Series Presentation, FSU, Tallahassee** 2008, 2010
  “Intracellular MRI Contrast by SPIOs at High Magnetic Fields”
“Bimodal Intracellular Nanoparticle Based on Quantum Dots for High Filed MR Microscopy”

- **Qualifying Examination in Biomedical Engineering, FSU, Tallahassee** 2007
  Review of “Dynamic Cell Seeding of Polymer Scaffolds for Cartilage Tissue”

- **Master Thesis Defense, Stockholm Sweden** 2005
  “Bioartificial Pancreas – Effects of alginate encapsulation on mitochondrial function and cell proliferation”

**PUBLISHED PAPERS**

**PRESENTATIONS, POSTERS and ABSTRACTS**


- Rosenberg, J.T.; Kogot, J.; Ridel C. Strouse, G. and Grant, S.C., Evaluation of Nanoparticle Contrast Agent Uptake in Murine Microglia (Bv-2) and Human Teracarcinoma (NT2) for Cell Tracking in Neurodegenerative Disease at 21.1 T, Joint Annual International Society for Magnetic Resonance in Medicine, Honolulu, Hawaii April (2009)


- Cornnell, H.H.; Grant, S.C.; Rosenberg, J.; Foroutan, P.; Sherry, A.D.; Edison, A.S. and Walter, G.A., Characterization of Paramagnetic Lanthanide Ion Complexes as MRI Contrast Agents as a

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Function of Magnetic Field Strength, Joint Annual International Society for Magnetic Resonance in Medicine, Berlin, Germany, May (2007)


COMPUTER SKILLS
- Good knowledge in MS Windows, MS Office, Para Vision 5.0 and 3.0, Top Spin, Amira 5.3.3
- Knowledge of MatLab and Pro Desktop (AutoCad)

SIGNIFICANT AWARDS AND HONORS:
- Royal Institute of Technology Study Scholarship for Material Science students 2005

GRADUATE AND POSTDOCTORAL ADVISORS:
- Master’s Thesis: Dr. Ioannis Constantinidis (U. Florida, Gainesville) and Prof. Håkan Elmqvist (Royal Institute of Technology, Stockholm, Sweden)
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    Dr. Teng Ma (FSU-FAMU, Tallahassee)
    Dr. Anant Paravastu (FSU-FAMU, Tallahassee)
    Dr. Geoffrey F. Strouse (FSU, Tallahassee)
    Dr. Cathy Levenson (FSU, Tallahassee)