Synthesis, Biofunctionalization, and Application of Magnetic Nanomaterials

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SYNTHESIS, BIOFUNCTIONALIZATION, AND APPLICATION OF MAGNETIC NANOMATERIALS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Materials Science and Engineering

by
Benjamin David Fellows
August 2018

Accepted by:
Dr. Olin Thompson Mefford, Committee Chair
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ABSTRACT

Since their inception in the late 1970’s magnetic nanomaterials have sparked heavy research into their use in the biomedical field. Their unique magnetic properties allow the magnetic particles to be the base for a large array of experimental medical techniques, from treatments of disease, diagnostic tests, imaging aids, and more. In this manuscript, each stage starting from novel particle synthesis, functionalization with bioactive molecules, and innovative application is explored, specifically using the techniques magnetically mediated energy delivery, magnetophoresis, magnetic resonance imaging.

The reproducible synthesis of nanomaterials is necessary if any further engineering application is going to be done. Using a novel extended LaMer approach where a precursor solution is consistently added to a reaction vessel allows for the linear volume growth of nanoparticles. This technique was originally used for the synthesis of magnetite (a simple ferrite) to control the volume of the particle indefinitely. Transferring it to a nonstoichiometric cobalt ferrite, it is shown that a linear volume growth is achieved up to 20nm.

Secondary functionality of the magnetic particles has really opened up the application to sensing, selective treatment, and functional imaging. Surface modification with a bacterial strain discriminatory glycan allows for strain selective treatment of bacterial infection. Heparin functional particles show high pharmacokinetic activity for the treatment of neointimal hyperplasia due to high surface area to volume ratios.
Gadolinium coated particles have distance dependent effects on the MRI relaxation rate of water, which may prove useful in functional imaging.

Each of these complexes shows promise as a new way to treat or image malady. Although a small part in the large picture in developing a new generation of medicine, this research lays the foundation for each of these possible treatments. Be it the eradication of bacterial infection or the non-toxic prevention of restenosis, novel multifunctional nanomaterials such as the ones discussed in this manuscript, will be heavily relied on in the future of medicine.
DEDICATION

I would specifically like to dedicate this to my Mother, Stephanie Fellows, who was my saving grace throughout all of the hardships endured during my graduate studies.
ACKNOWLEDGMENTS

First I would like to acknowledge my PhD committee members, including my advisor Dr. Olin Thompson Mefford, Dr. Igor Luzinov, Dr. Marek Urban, and Dr. Delphine Dean. They have been extremely helpful and supportive with their feedback, their discussion, and their patience. I would also like to acknowledge all of the excellent graduate and incredible undergraduates that I have worked with throughout the years, specifically, Nardine Ghobrial, Yash Raval, Elliot Mappus, Andrew Hargett, Kristin Fuller, Sarah Sandler, Jessica Bigner, Yves Cordeau, Jamie Murbach, and Will Glasgow.

I would also like to thank my friends who have helped me through the journey to a PhD including Dmitriy Davydovich, Eric Zhang, Matt Tuggle, Benoit Faugas, Maxime Cavillon, Dr. Katie Davis, Tucker McFarland, my literal cat Oliver Fellows and everyone else who has made my stay here unforgettable.

I would like to acknowledge both Dr. Gary Lickfield, and Bob Bowen for their guidance and the unbelievable tailgates with materials research society.

Lastly I would like to thank my family especially my mother Stephanie Fellows, my father John Fellows, my brother Gabe Fellows, my aunt and uncle Katie and Steve Deren, and my grandparents Jane and Charles “Chuck” Gulling whom I owe for the slightly larger cardboard box I’m living in. Thank you all for everything you have done for me during my time here at Clemson.
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CHAPTER ONE

Introduction to Magnetic Nanomaterials in Medicine

Magnetic nanomaterials have grown ubiquitous in the biomedical field for their wide variety of applications ranging from basic transfection promoters to targeted disease treatment and theragnostics. This large body of applications is only possible due to the unique properties of nanoscale magnetic particles which is important to understand when discussing their use. Although there is a large number of magnetic materials where these properties manifest at the nanoscale, one particular class, ferrites, are heavily used in the medical field due to their low toxicity, desirable magnetic properties at body temperature and high versatility.¹

Within ferrite materials the most commonly studied and implemented in material is magnetite (Fe₃O₄) which has a relatively high magnetic saturation, with bulk being 90 emu/g and particle saturation magnetization between 30-90 emu/g, and is one of the simple ferrites as it contains only iron in both the 2+ and 3+ oxidation state.² For the sake of simplicity, this introduction will focus on magnetite as the primary example of magnetic nanoparticles when looking at important magnetic properties and their manifestation in biomedical applications.
Figure 1. Medical applications relying on the magnetic properties of nanoparticles. i) In MRI, magnetic nanoparticles affect the magnetic relaxation rates of hydrogen atoms in each tissue type, resulting in a contrast in MRI. ii) Applying an alternating magnetic field to magnetic nanoparticles can induce heat generation. The resulting temperature increase in tissue can induce tumor cell death and/or enable temperature-controlled drug release. iii) Drug-loaded magnetic nanoparticles accumulated at a target site by a magnetic attraction force. iv) Magnetic removal of pathogens and/or circulating tumor cells as a potential new therapy that differs from drug administration and surgical intervention. Hyek Jin Kwon, Kwangsoo Shin, Min Soh, Hogeun Chang, Jonghoon Kim, Jisoo Lee, Giho Ko, Byung Hyo Kim, Taeghwan Hyeon: Large-Scale Synthesis and Medical Applications of Uniform-Sized Metal Oxide Nanoparticles. Advanced Materials. 2018, Online early view, Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.
The magnetics of the particles depend on multiple different variables including the magnetocrystalline anisotropy and the magnetic moment. Each of these can be controlled via particle synthesis, but it is important to differentiate the method of control. Magnetocrystalline anisotropy defines the energy requirements to magnetize along different crystal lattice axes. This means that there exists an “easy axes” where the moment is preferentially aligned and at its lowest energy state, as well as a “hard axes” which takes a large amount of energy to magnetize along. This anisotropy magnitude and direction are both inherent to the material in question meaning that each chemical makeup of a material has a unique constant that defines this. To change this value, it is necessary to change the actual chemical formula and crystalline structure of the material. It is true that this magnetocrystalline anisotropy value does not describe the whole anisotropy contribution of the particle system as it does not include surface, defect, or shape effects. It is arguably the largest contributor to this total anisotropy value and therefore an important variable to consider.

The magnetic moment of the particles is representative of the magnetic strength and orientation of the field produced for the particles. The moment’s magnitude is limited by the saturation magnetization of the material. This is an important value as it partially describes the forces and behavior that particle may respond to inside of an applied field and field gradient. This total moment is highly dependent on particle radius and the inherent magnetism exhibited whether it is above or below the blocking temperature of the particle. Particles on the nanometer scale may exhibit superparamagnetic behavior above their blocking temperature which is defined where $\tau_m = \tau_0$ in equation 1, where $T_B$
is the temperature, $K$ the anisotropy energy density, $V$ is the particle volume, $k_B$ is Boltzmann constant, $\tau_m$ is the measurement time, and $\tau_0$ is attempt period.

$$\text{Eq. 1} \quad T_B = \frac{KV}{k_B \ln(\tau_m/\tau_0)}$$

When the Néel relaxation time (attempt period) is much less than the measurement time, the moment is random and so net magnetization is zero, this is known as the super paramagnetic regime. As the Néel relaxation time approaches the measurement time, the moment cannot relax so a measurable magnetization is observed. The temperature at which this switch occurs is called the blocking temperature. Essentially the net moment of a superparamagnetic particle solution is zero without a field. This is due to adequate thermal energy overcoming any dipole and anisotropic energy limits allowing the single moments to orient randomly. In the presence of an external field however, the particle moment will align parallel with the field. Superparamagnetism is also associated with a zero magnitude magnetic hysteresis when measured with relatively slow measurement times ($< \text{kHz}$). Thermal fluctuation and magnetic relaxation to the easy axis via internal electronic Néel relaxation or viscous physical Brownian relaxation is adequately fast that a zero hysteresis is measured. Typical DC magnetization vs. field loops have a slow sample rate and therefore show this curve which has become standard for showing superparamagnetic behavior.
Figure 2. A) Example magnetization vs. field loop showing superparamagnetic behavior when measured in a DC magnetometer. Note the lack of hysteresis. B) Zero field cooled-field cooled loop showing the blocking temperature of the particles at approximately 250K.

The distinct magnetic properties of the particles allow for their use in multiple different biomedical applications. This includes but is not limited to magnetically mediated energy delivery (MagMED), magnetophoretic targeting, magnetic resonance imaging contrast, biosensing, magnetic particle imaging, and many others. Specifically, MagMED, magnetophoretic targeting, and MRI contrast are of particular interest and will be discussed in depth.

Magnetically Mediated Energy Delivery

Magnetically mediated energy delivery (MagMED), formally known as magnetic field hyperthermia, has been a heavily researched area in the magnetic community for many years. The concept behind this technology is that an alternating magnetic field is
applied to particles in and around a specific cell population. The particles convert the energy of the magnetic field and deliver it locally to the cells.\textsuperscript{8,9} It was thought that the energy transfer potentially causes a local elevation in temperature respective to the magnetic materials, which can exceed the feasible temperature limit of the target tissue causing cell death.\textsuperscript{10-14} However, the complex nature of this energy transfer is poorly understood, as several groups have demonstrated cell death with no bulk heating.\textsuperscript{15-20} Because of the misnomer, MagMED is now the preferred term of this group and others for what was previously known as magnetic field hyperthermia.

The idea of locally induced energy delivery goes back to 1957 with the direct injection of maghemite ($\gamma$-Fe$_2$O$_3$) into tumorous lymph nodes followed by treatment with an alternating magnetic field.\textsuperscript{21} Since then, the idea of local heating has attracted both research and clinical interest for its ease of treatment, efficacy, and low degree of invasiveness.\textsuperscript{22} Traditionally, this work has been centered on iron oxides including the most commonly studied phases, maghemite ($\gamma$-Fe$_2$O$_3$) and magnetite (Fe$_3$O$_4$). Much of this research is directed towards the synthesis, characterization, and application of iron oxides. Despite significant research activity, there are limited commercial and clinical applications of these materials to date. One reason for this is the limited properties of Fe-ferrite materials.

More recently, to improve upon the material properties of iron oxides, researchers have begun to consider substituted and doped ferrites. These complex materials show a wide range of magnetic properties, including tunable magnetic saturation, magnetocrystalline anisotropy, and blocking temperature.\textsuperscript{23} Coupled with recent
advancements in synthesis, and increasing control over both size and morphology of nanoscale colloids, these new materials have been shown to exhibit properties that are on the cusp of being clinically relevant for MagMED.

Figure 3. Representative figure showing the calorimetry setup for SAR values. A) Optical thermocouple submerged in solution to measure heating. B) Typical time vs temperature graph, note the initial slope is used for SAR calculations. C) Hollow induction coil with water coolant. D) Water jacketed sample holder for equilibration of sample temperature before measurement. E) Sample enclosure and cap are necessary for sample manipulation and to reduce evaporation. F) Known volume of sample used (in this case 0.5ml of toluene). G) Illustration of moment switching with the AC field causing energy transfer from the particles.

MagMED treatments primarily rely on the optimization of one parameter, the specific absorption rate (SAR), which describes the efficiency of energy conversion at a
The SAR parameter is normally measured by inserting a known concentration of particles into a coil and observing the change in temperature as a function of time of the colloid or solid sample. This value can then be used to calculate SAR for the material in question as is seen in equation 2. SAR is reported as the power per unit mass of sample, \( C_p \) is the specific heat capacity of the colloid, \( \Delta T/\Delta t \) is the measured slope of the time dependent heating curve, and \( \varphi \) is the mass fraction of the material in \( g_{\text{material}}/g_{\text{solution}} \).

\[
\text{Eq. 2 } \text{SAR} = \frac{C_p}{\varphi} \frac{\Delta T}{\Delta t}
\]

Although this is the most common way the SAR values are calculated, this method does not account for both the field and frequency specific contributions of the energy transfer. Some groups have argued to normalize this value by dividing by the frequency and the field squared. However, as discussed below, the field and frequency dependency makes this an inherently flawed normalization. Nonetheless, the energy produced for each cycle can be described by the hysteresis loop area of the particles in the AC field as:

\[
\text{Eq. 3 } A = \int_{-H_{\text{min}}}^{+H_{\text{max}}} \mu_0 M(H) dH
\]

where \( A \) is the energy released, \( M(H) \) is the field dependent magnetization and \( \mu_0(H_{\text{max}}) \) is the field amplitude. It should be noted that this loop is often different to one measured
using traditional magnetometry techniques that have much longer measurement times. Put a different way, hysteresis loops measured using traditional magnetometry, i.e. vibrating sample magnetometry with a superconducting quantum interference device (SQUID) detector, do not correctly represent behavior at the field and frequencies of MagMED.
Figure 4. Evolution of the hysteresis loop as a function of field frequency and characteristic particle relaxation time showing there exists optimal conditions for each particle system to get the largest energy transfer. Too low a frequency and the particles behave as paramagnets, too high a frequency and the particles are never directionally magnetized. Figure was taken from J. Carrey, B. Mehdaoui, M. Respaud, Simple models for dynamic hysteresis loop calculations of magnetic single-domain nanoparticles: Application to magnetic hyperthermia optimization Journal of Applied Physics 2011, 109, 083921. Figure was reprinted with permissions from AIP Publishing.

Nonetheless, the time dependent energy release rate can then be multiplied by the frequency \( f \) to give the SAR value (equation 3).\(^{24}\)
The critical value in equation 4 is the energy/heat generation per cycle, and it is this value that materials chemists are able to greatly influence through tuning physical-chemical parameters relating to the energy transfer mechanism. This mechanism results from hysteretic loss which has been described for single domain magnetic nanoparticles above the ferromagnetic transition point.\textsuperscript{24, 27, 28} At smaller volumes, using the linear response theory this hysteretic loss mechanism occurs under the superparamagnetic threshold value where particle sizes are much smaller than their ferromagnetic counterparts. In this case, the combined Néel and Brownian relaxation time as a function of frequency contributes to the energy loss. By matching the frequency with the specific loss peak it is possible to optimize small particles under their ferromagnetic threshold. This size dependent response emphasizes the importance of the magnetic volume on the resulting properties. More so, different particle sizes strongly vary the properties of biodistribution, circulation time, and clearance.\textsuperscript{29, 30} This superparamagnetic-ferromagnetic transition volume is material specific, thus it may be possible to create particles that both exhibit hysteretic loss and have the distribution properties of their smaller counterparts.
Nanoparticles for Magnetophoretic Targeting

Magnetophoretic mobility and targeting is a technique that used the high saturation magnetization of the particles to magnetically attract the particles to a specified area via a large induce field gradient. This method of targeting is attractive for \textit{in vivo} use because the particles will only respond to an externally applied field which can be specific to an anatomical region or induced in magnetizable implants.\textsuperscript{31-35} Particles once outside of the targeted field region will revert back to a random state and maintain stability until either they are cleared biologically or circulated and see the field again. This is important as it makes follow up treatments minimally invasive and allows for non-specific biological moieties to be selectively targeted, increasing accumulation at the target site and reducing the possibility of systemic side effects that would occur in typical administration.\textsuperscript{36}

The efficacy of this technique is highly dependent on many factors including the particle magnetization, field gradient, and surface chemistry of the stabilizing ligand. The gradient and moment of the particle must be sufficient enough that it overcomes both the stabilization of the particle in solution as well as viscosity effects from the ligand interaction within the solvent.\textsuperscript{37-40} It is also important to note that most applications \textit{in vivo} require the targeting to happen in some vascularized tissue. This is not a static environment and so there exists a flow rate which gives the particles a small time window to be captured. Again this may be expanded through either multiple administrations or bioinert surface chemistry which impart a “stealth” property by manipulating the protein
corona, increasing their biological half-life essentially allowing them multiple passes through the target site. 

Currently the use of the magnetophoretic properties is gaining interest in externally directed drug delivery and cell transport. This includes much research into using magnetite decorated endothelial cells to reduce the probability of in-stent restenosis. This treatment is surprisingly effective considering the forces needed to significantly move cellular organisms. The magnetic force created is from an implantable stent is a function of the particle magnetic moment ($\vec{m}$), the field gradient due to the stent struts ($\nabla \vec{B}_g$), and the externally applied homogeneous magnetic field ($\vec{B}_0$).

$$\text{Eqn. 3 } \vec{F}_{mag} = (\vec{m} \cdot \nabla \vec{B}_g)\vec{B}_0$$

The importance of both the magnetic moment of the particle, and the field gradient created from the magnetic stent struts are seen in equation 3. This illustrates the need for high magnetic saturation particles as well as a highly magnetizable stent. Another option that may be used to increase the gradient would be to replace the homogeneous field with a large field gradient. Issues in uneven targeting may occur as the particles will be forced down the external gradient.

Nanoparticles in MRI

The use of magnetic nanoparticles as magnetic resonance imaging (MRI) contrast agents has been of particular importance and is one of the most heavily researched
properties, with agents going so far as being approved for medical use by the United Stated Food and Drug Administration.\textsuperscript{42} With the implementation of MRI in the 1970’s and 80’s, came an influx of contrast related research. Ferrite particles were seen to impart large changes in T2 relaxation rates, and started being evaluated as diagnostic tools as early as 1986.\textsuperscript{43} Since their inception, contrast agents have been crucial in the diagnostic field as they allow clinicians to differentiate between tissues or fluids in the body that may have similar relaxation signals. These agents are typically split into two different categories, T1 and T2 agents.
Figure 5. Illustration of T1 and T2 contrast effects. T1 agents brighten as concentration increases, where T2 agents darken with concentration.

This refers to the mechanism of relaxation that they predominately change. In magnetic resonance, each unique proton in the applied field is aligned either parallel or antiparallel to the applied field. Due to Zeeman splitting there is a degeneracy, meaning that there are a slightly disproportionate number of spins aligned parallel to the field. Each of these spins processes around the field vector at a rate defined by the field magnitude and chemical environment of the proton. This procession is named the Larmor frequency. For T2 in a thermally equilibrated system there is no phase coherence between moments, so no signal can be measured. When a specific radio frequency pulse is applied to the system, the magnetization vector of the protons is shifted into the X, Y plane from the initial magnetization along the Z axis.
Figure 6. T2 relaxation mechanism showing net moment along z axis, phasing 90 degree pulse and dephasing of the spins. X axis magnetization (signal) is shown below spin diagrams illustrating why T2 is inherently negative contrast or signal loss.

The T2 relaxation is defined by the dephasing time of the RF phased magnetic moments which is constituted by a loss in signal magnitude in the X-Y plane. This dephasing is defined by equations 6 and 7 below where $M_x$ and $M_y$ are magnetization in the respective planes, $M_0$ is the initial magnetization, $t$ is the time, $T_2$ is the relaxation and $\omega$ is the frequency of procession. Typically the T2 relaxation is defined as the time when the signal magnitude is equal to 1/e.44

Eq. 6 $M_x(t) = M_0 e^{-\frac{t}{T_2}}Sin(\omega t)$

Eq. 7 $M_y(t) = M_0 e^{-\frac{t}{T_2}}Cos(\omega t)$
Because T2 based contrast agents lower the time it takes for the signals to dephase, it is known as negative contrast enhancement essentially making the image taken from the MRI appear darker when compared to a control image. Magnetic nanoparticles are well defined as T2 contrast agents as they induce large changes in the T2 relaxation mechanism. Because of the large local field generated by the particles, large differences in the field gradients are felt by nearby protons changing each unique procession frequency. This in turn accelerates dephasing as the field defining the Larmor frequency ($\omega_i$) of each proton is now a function of summation of the applied field ($B_0$) and the local field from the particle ($B_p$) as seen in equation 8.

$$\text{Eq. 8 } B_0 + B_p = B_{tot} \propto \omega_i$$

Although T2 is not preferred by most clinicians, as it is negative contrast, there are very large changes in the hyperintensity signal produced by the magnetic particles. Typical changes in T2 signals generated by magnetite nanoparticle based contrast agents are an order of magnitude greater than their gadolinium counterparts. The large T2 effect means that the T2 signal will overwhelm any T1 weighted signal from the particles, so positive contrast T1 imaging is difficult. Although not typically used in positive contrast T1 weighted scans, the large T2 effect allows for magnetite nanoparticles to find niche uses in diagnostics for mainly hepatic ailments where particles gather quickly at the target site. Although promising, there have been some issues with
the particles for T2 contrast agents as they have been seen to cause adverse effects in some patients including cardiovascular distress and lower back pain.\textsuperscript{47} This may be due to the propensity of the particles to form long mesoscale chains over time in a magnetic field which was shown by Saville et al.\textsuperscript{48} Any magnetic interactions between particles are essential to suppress as aggregation in vivo may cause changes in the behavior of the material. This again alludes to the surface chemistry playing a large role in the stability and even magnetics of the particles, as a larger more steric surface chemistry may delay or hinder chaining from occurring on the timescale of measurement.

Although magnetic nanoparticles are not typically used as T1 contrast agents, it is important to distinguish the difference in the relaxation mechanism from T2 as there is research into possibly using them particles as dual contrast agents. The T1 phenomena is the relaxation back to thermal equilibrium in the z direction after a “demagnetizing” pulse is applied. Because you are looking at the regain of signal it is inherently a positive contrast mechanism, which shows up as bright spots on typical MRI scans.
Figure 7. T1 relaxation mechanism showing initial signal from net moment along z axis, 90 degree pulse and Relaxation back to thermal equilibrium. Z axis magnetization (signal) is shown below spin diagrams illustrating why T1 is inherently positive contrast or regain in signal.

This is much easier to distinguish than its T2 counterpart in an image readout. Unlike typical T1 contrast agents, which are made up of chelated paramagnetic metal ions like gadolinium, the ratio of T2 to T1 of a magnetic nanoparticle is so high that the T2 signal swamps any T1 weighted measurement which does not allow particles to be used at T1 contrast agents on their own. The idea to use dual weighted T1/T2 imaging is ideal as it can eliminate artifacts unique to each of the different imaging modalities using an AND gate which compares the images to see if contrast is present in both images. This method of imaging relies on the fact that the T2 signal from the particles will not
influence the T1 weighted imaging. Changes in the particle size and surface
functionalization can affect the particle specific relaxation rates. This can amount to
lowering the T2 and increasing the T1 so that dual mode imaging is viable.

Surface Chemistry

Just as important as the magnetic properties of the particle is the surface
chemistry, which imparts stability, functionality, and biocompatibility. Without a robust
chemistry, the particles are not suitable for use in vivo as they may lose potency, and
potentially pose a significant health risk. Many studies have looked at the toxicology of
nanomaterials in general and it is agreed upon that the surface chemistry, and access to
particle crystal faces plays a large part in determining the overall toxicity. Not only is
the toxicity of magnetic nanomaterials highly dependent on the surface chemistry, the
magnetics are also affected. Because magnetite is relatively benign and is metabolized
into useful iron, toxicity and metal ion leaching or degradation in vivo is not nearly the
issue it is with other substituted metal ferrites e.g., CoFe$_2$O$_4$, NiFe$_2$O$_4$, or MnFe$_2$O$_4$. Although magnetite may not have inherently toxic metals, the system still may lose
potency if leaching occurs, and it serves as a model system for other mixed ferrite
systems, as good binding moieties for magnetite carryover to the other ferrites.

The method of particle synthesis changes the method and ease of attaching
biologically stable ligands. “Nude” particles synthesized via coprecipitation are charge
stabilized so surface modification is relatively easy as there are no competing ligands to
bind to the surface or replace. Unfortunately, coprecipitation, although easy, gives
particles that are typically polydispersed and irregular in morphology. For many applications it is important to control the size and shape to a high degree. This is much easier done via thermal decomposition of organometallic precursors. The tradeoff for this control is that they are typically sterically stabilized via hydrophobic ligands. These are more difficult to replace as they are already bound to the magnetic particles and, even after purification, present in heavy excess. The common method for modification to a biocompatible material involves ligand exchange which uses an energy source, commonly sonication, to increase the exchange of ligands. By introducing a competing ligand with higher binding affinity, overtime the particles should be preferentially coated with the new ligand.

A large amount of time and effort has gone into identifying high affinity organic moieties to bind to the surface of magnetic nanomaterials. Davis et al. have shown that there are effectively two binding groups, catechol and phosphonate, which have the highest affinity for ferrites and should be used for ligand binding or ligand exchange procedures as seen in figure 8.54
Figure 8. Chains of oleic acid remaining on the surface of the nanoparticles before and after ligand exchange determined by LSC. The data for the PEG-coated nanoparticles represent the amount of oleic acid remaining after dialysis. The lower the chains of oleic acid the greater the extent of ligand exchange. Reprinted (adapted) with permission from Quantitative Measurement of Ligand Exchange on Iron Oxides via Radiolabeled Oleic Acid, Kathleen Davis, Bin Qi, Michael Witmer, Christopher L. Kitchens, Brian A. Powell, and O. Thompson Mefford, Langmuir 2014 30 (36), 10918-10925, DOI: 10.1021/la502204g. Copyright (2014) American Chemical Society.

These moieties are typical in literature and are commonly used as the anchoring site for large macromolecules which further stabilize, and disperse the particle in aqueous media.
Although the binding affinity of these groups is so high, it is not uncommon to see difficulties in maintaining a stable colloid under biological conditions. This is due to the relatively large amounts of both salts and proteins in typical human serum as well as possible competing ligands in biological systems. One effective strategy to avoid possible surface polymer detachment is to have multiple anchor points for each macro molecule. Saville et al. have shown that by increasing the number of catechol binding groups from one to three on the end of a stabilizing macromolecule, the particles maintain stability in not just high salt content solutions of phosphate buffered saline but high protein solutions of fetal bovine serum as well. This is due to the difficulty of detaching the ligands that are bound in triplicate to the particles. The stability conferred by this multiple binding scheme is of the utmost importance as destabilization of the particles, due to unwanted ligand exchange and aggregation, does not occur and the particles can be suspended.
One of the most common macromolecule used in the biocompatibility and stability process is poly(ethylene oxide) (PEO) also referred to as poly(ethylene glycol) (PEG). PEG is a simple polyether that is bioinert and nontoxic when the molecular weight is above a threshold value. Although water is not an ideal solvent, it solvates and disperses relatively well. PEG is commonly used in the pharmaceutical industry to aid delivery and increase circulation times of hydrophobic drugs, and is even approved for use as a laxative as it solvates and retains water, but does not interact with electrolytes. This same mechanism is what makes PEG an optimal coating for nanoparticles in general as it interacts with the water but is relatively inert to ions and pH changes.\textsuperscript{56} Other surface
coatings have been explored including dextran, which is a polysaccharide that imparts stability, and albumin, which is a biologically derived protein, but these coatings do not have the low interaction potential that PEG does due to the large amount of functional groups contained in the polymer.\textsuperscript{57}

PEGs used to impart stability in particle systems are typically multifunctional, meaning they have two different endgroups where they can be further modified. Typically one end will be composed of the anchoring group (typically a catechol or phosphonate derivative). This group binds to the particle attaching the PEG to the surface. The other endgroup is typically tailored so it can be reacted with biomolecules that give more functionality to the particles. This can be a targeting molecule such as an antibody or glycan, a pharmaceutical, e.g. heparin, or other moieties that impart secondary functionality or change the behavior of the particle e.g. Contrast agents, clustering particles through crosslinking etc.\textsuperscript{58-61} This ability to conjugate any number of other molecules to the surface, greatly enhances the prospective uses of the particles for biomedical application. Another common modification is using PEG and PEG block-copolymers to impart release conditions of a drug be it temperature, pH, or some other outside stimulus. Typically the PEG block will be on the surface to impart the stability where the stimuli reactive block will be the intermediary between the particle and the PEG coating.\textsuperscript{62} These intermediary blocks can be made up of different polymers, and is typically where the drug of choice is loaded.\textsuperscript{63}
Magnetic Particle Synthesis

In recent years a large amount of research has gone into developing highly controllable synthesis methods for nanoscale colloids. This is especially true with magnetic nanoparticles, as they have been one of the most sought after nanoscale materials. Because of this, the various synthesis methods of metal ferrite nanoparticles have grown both simpler and more reproducible. It is now possible through a variety of methods to obtain monodispersed nanoparticle populations, phase pure crystallites, controllable morphologies, and a range of sizes. This is important, as requirements for the optimization of particle systems for use in MagMED, sensing, and many other applications are stringent, and even slight deviations from these parameters can have a drastic effect the efficacy of the particles.

Of the vast array of synthesis methods, a few are more commonly used and seem to yield the best results for tuning particle parameters important for their use in the biomedical field. The first and most recognizable is coprecipitation. In essence, the synthesis uses a variety of metal salts containing both an Fe$^{3+}$ cation and a mixture of M$^{2+}$ cations (M = Mn, Zn, Fe, Co, Ni, etc.) dissolved in an aqueous system.
Figure 10. Coprecipitation synthesis of magnetic nanoparticles, with representative image of particles produced. Although the reaction is simple, control over morphology and size is minimal.

This can be done in the presence of a stabilizing surfactant that will eventually cover and sterically or electrostatically stabilize the particles. However, inclusion of surfactant is not necessary as bare particles resulting from the reaction will have charged surface groups granting them stability at defined pH conditions. Normally, the precipitation of the nanocrystals takes place after a base is added and instabilities in the system cause the nanoscale crystallites to crash out of the solution creating an emulsion. These are then separated from the supernatant by antisolvent precipitation or magnetic separation and carefully washed to remove excess salts, surfactants, etc. It is imperative that the pH values of the solution are carefully monitored, as coprecipitation particles are prone to agglomeration. Variables contributing to the particle parameters include the specific anion chosen, reaction temperature, stirring rate, pH value and the ionic strength of the solution.64
Coprecipitation particles are widely used as their synthesis is relatively easy, and the particles as synthesized are water suspendable. However, difficulties in controlling the exact morphology, wide size distributions, and potential stability issues are drawbacks of the coprecipitation synthesis method.\textsuperscript{64, 65}

The second widely used method is thermal decomposition. This method of synthesis allows for a high degree of control over particle size and dispersion.\textsuperscript{64, 66} This is important when considering techniques that require more stringent magnetic parameters like MagMED, as it allows for more precise tuning of size and morphology both of which contribute to the overall SAR of the material.\textsuperscript{24, 67} It has also been shown in literature that various more complex materials such as doped ferrites and core shell particles are able to be synthesized this way.\textsuperscript{66} This method of preparation uses an organometallic precursor for the metal ion source.

![Figure 11. Example thermal decomposition synthesis reaction for magnetic nanoparticles. Oleic acid is the stabilizing ligand which is coating the particles. Although more difficult, high control of particle size and morphology is achieved.](image-url)
The precursor is normally mixed in with both a high boiling point organic solvent and an organic capping ligand (the most common being oleic acid). The reaction is run at high temperatures for varying amounts of time causing the organometallic precursor to decompose, yielding a solution of free metal ions. At a critical supersaturation point nuclei crash out of solution and the particles go through a growth phase. This growth phase can be controlled by constant introduction of the metal precursor, effectively regulating the amount of free iron available for the particles. Cooley et al. demonstrate an “extended LaMer” approach and show incredibly high degrees of size control with low size dispersion. When the reaction is cooled the resulting particles are homogeneous in size and shape, but they are rendered insoluble in aqueous solutions due to the long chain fatty acid stabilizing ligand.

The non-polar surface coating of oleic acid is problematic when considering particle use for MagMED, as the particles as synthesized are not biologically viable. Much work has been done in looking at the post modification of oleic acid coated particles by encapsulation or ligand exchange with biocompatible macromolecules such as poly(ethylene glycol), bovine serum albumin, dextran, etc. to overcome the compatibility issues. Another issue relating mainly to metal ferrites is in the variable decomposition temperatures of different organometallic precursors. These differences play a large role in determining the chemical makeup of the particles, and can be attributed to unexpected morphologies obtained. Although initially perplexing, compositional dependence on chosen precursor for thermal decomposition is promising and may be exploited in future
work to obtain varying degrees of substitution while retaining the advantages in particle size control and a low degree of dispersion.
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CHAPTER TWO
SYNTHESIS OF SUBSTITUTED METAL FERRITE NANOPARTICLES

Introduction

Addressing the challenges of MagMED from a materials perspective is done primarily the initial particle synthesis. This is where the anisotropy, and the particle volume can be explored and optimized. In this study we look at multiple transition metal substituted ferrites and their utility as a MagMED platform. Using a one pot synthesis, chosen compositions were screened, using specific absorption rate as a measurement of efficacy in energy transfer. Energy-dispersive X-ray mapping was used to investigate the elemental composition and morphology.

From the initial one pot synthesis and characterization of the doped metal ferrites, the doped cobalt ferrite had the largest SAR value, as well as homogenous loading throughout the lattice of the substituted cobalt. This along with cobalt ferrites already high anisotropy led us to focus on the cobalt system. Traditionally, research in MagMED has been centered on the most commonly studied iron oxide phases, maghemite ($\gamma$-$\text{Fe}_2\text{O}_3$) and magnetite ($\text{Fe}_3\text{O}_4$). Despite significant research activity, there are currently limited commercial and clinical applications of these materials. Recently, to improve upon the material properties of iron oxides, researchers have begun to consider substituted and doped ferrites\textsuperscript{1-5}. These materials show a wide range of magnetic properties, including, tunable magnetic saturation, magnetocrystalline anisotropy, and blocking temperature.\textsuperscript{6} Coupled with recent advancements in synthesis, and increasing control over both size and morphology of nanoscale colloids, these new materials have been shown to exhibit
properties that are greatly improved from those of Fe-ferrites. One of these select materials is cobalt ferrite (CoFe$_2$O$_4$), which has a much greater effective anisotropy due to the replacement of the Fe$^{2+}$ with the highly anisotropic Co$^{2+}$ ions.$^7, 8$ By maximizing the effective anisotropy through Co doping, specifically between cobalt levels between 0.4 and 1 in Co$_x$Fe$_{3-x}$O$_4$, as well as identifying the optimal volume for energy release using a novel extended LaMer synthesis, the optimization of cobalt ferrite based materials for application in MagMED is possible.$^9, 10$ Herein, we describe a potential synthetic method to produce these materials in a size controllable manor, and the resulting properties of the particles relating to MagMED.

Experimental

One-Pot Thermal Decomposition Synthesis

Initial investigation of doping effects was done using a one-pot synthesis of cobalt ferrite particles. Stoichiometric amounts of both iron (III) acetylacetonate and cobalt (II) acetylacetonate were combined in a 100 ml 3-neck round bottom flask, for a total amount of 3.04 mmols of organometallic precursor. Oleic acid (15 ml) was then added, serving both as the solvent and primary ligand for particle stability. The vial containing the solution was placed into a metal bath at 200 °C, nitrogen purged, and then quickly ramped to 350 °C, to avoid heating rate variance. The solution was left to sit, under nitrogen and stirring with an overhead stirrer, for 3 hours. Samples were precipitated with an ethanol: acetone mixture (3:1) (3x) and resuspended in toluene. The samples were run
through an organic based gel permeation chromatography (GPC) column to purify off excess oleic acid and unreacted organometallic precursor\textsuperscript{11}.

Metal Oleate Precursor Synthesis

Metal oleates were synthesized by combining 9.3 mmol of metal acetylacetonate and 15 ml of oleic acid in a 100 ml 3-neck round bottom flask. The reaction was initially purged and performed under constant (<50 ml/min) nitrogen flow. After adequate purging of the flask, it was heated to 320 °C for 20 minutes and then quenched by removing the heating source, yielding a dark, oily liquid that did not respond to an external magnet. Fourier-transform infrared spectroscopy was performed to confirm the presence of the oleate complex with the minimization of the free acid peak at 1710 cm\textsuperscript{-1} and the appearance of the carboxylate anion with peaks at 1578 cm\textsuperscript{-1} and 1440 cm\textsuperscript{-1} shown in figure 1.

![Figure 1. FT-IR of one-pot synthesized metal oleate precursors for extended LaMer.](image)

\textit{Figure 1. FT-IR of one-pot synthesized metal oleate precursors for extended LaMer.}
Drip Thermal Decomposition Synthesis

The metal oleate precursors were diluted to 0.22 M in octadecene to help with injection into the reaction flask. Diluted precursors were combined in specific predetermined molar ratios and vortexed to ensure homogeneity. The precursor mixture was loaded into a 10 ml syringe attached to a penetration needle, and affixed to a syringe pump. The 3-neck flask was loaded with 2.5 g of docosane and 2.5 ml of oleic acid and heated to 350 °C under inert (nitrogen) atmosphere. When the solution temperature was stable, the precursor solution was added at a rate of 3 ml/hr. Aliquots were started 20 minutes after the first addition of the precursor solution, and were taken every 5 minutes for 90 minutes.

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was done using a Thermo-Nicolet Magna 550 FTIR spectrometer. 16 Scans were done for both the sample and the background. Samples were prepped by dropping a small amount of the oleate sample onto a diamond crystal. Crystal contact was assumed to be good due to the liquid nature of the sample.

Transmission Electron Microscopy

Bright field transmission electron microscopy was run on a Hitachi H7600 with a variable spot size. Image analysis was done using Image J (NIH, open-source) on a
minimum of 300 particles. High Angle Annular Dark Field Scanning Transmission Electron Microscopy (HAADF-STEM) imaging and energy-dispersive X-ray element mapping were carried out using a FEI Titan G2 80-200 TEM/STEM with ChemiSTEM Technology operating at 200 kV. The element maps were obtained by energy dispersive X-ray spectroscopy using the Super-X detector on the Titan with a probe size ~1 nm and a probe current of ~0.9 nA.

**AC Magnetometry**

Volume AC magnetometry was run on an Imego Dynomag. 200 µl samples of particles suspended in toluene were pipetted into a 1ml borosilicate glass vial. Peak frequency was extrapolated from a best fit imaginary susceptibility curve.

**AC Calorimetry**

Specific absorption rate (SAR) measurements on particles were done using a field of 38 kAm$^{-1}$ at a frequency of 206 kHz. A sample containing 500 µl of purified particles in toluene were transferred into a 1ml glass vial. The vial was inserted into the water jacket contained within the coil and an optical temperature probe submerged into the toluene solution. Toluene was used to minimize evaporation between runs. The water jacket temperature was set to 37 °C. Once the sample temperature was stable, the power source was turned on and run for 120 s. The rise in temperature was recorded as a
function of time and the initial slope based on the first 40 seconds of heating was used in calculating the specific absorption rate.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

ICP-OES was run using a Perkin Elmer Optima 3100 ICP-OES running WinLab32 version 3.1 analysis software. Particle samples were digested initially with 30% hydrogen peroxide solution (Sigma-Aldrich) to remove organic. The peroxides were boiled off and the remaining inorganic was digested with <70% nitric acid (ARISTAR PLUS, VWR). The nitric acid was boiled off and the resulting nitrate salts were dissolved in a known mass of 2% nitric acid solution and run against standard solutions.

Thermal Gravimetric Analysis

Thermogravimetric analysis (TGA) was used to investigate decomposition temperatures of metal acetylacetonates. 5 to 10 mg of each sample was placed in a TGA pan, which was analyzed using a TA Instruments 2950 TGA. The samples were heated at 20 °C/minute under nitrogen purge to 110 °C, held at 110 °C for 30 minutes, then heated at 15 °C/minute to either 500 °C or 800 °C.

Small-angle X-ray Scattering

Small-angle X-ray scattering (SAXS) experiments were conducted using a SAXS LAB Ganesha at the South Carolina SAXS Collaborative at the University of South
Carolina. A Xenocs GeniX3D microfocus source was used with a copper target to generate a monochromatic beam with a 0.154 nm wavelength. The instrument was calibrated using silicon powder (NIST 640e). Scattering data were processed from the scattering vector $q = 4\pi \lambda^{-1} \sin \theta$ where $\lambda$ is the X-ray wavelength and $2\theta$ is the total scattering angle. A 300K Pilatus detector (Dectris) was used to collect the two-dimensional (2D) scattering patterns. SAXS GUI software was used for radial integration of the acquired 2D patterns to reduce the data to 1D profiles. Sample solutions were prepared by diluting the crude reaction product to <5 vol% in hexane to avoid structure factor contributions to the scattering curve.

Samples were passed through a 200 μm syringe filter just prior to measurement in a sealed glass capillary. A blank sample consisting of a capillary with only hexanes was measured under the same conditions for background subtraction using SAXS GUI. All samples were acquired for 15 minutes at room temperature with an incident X-ray flux of $\sim 21.4$ M photons per second.

Small Angle X-ray Fitting

Nanoparticle dimension distributions were determined by fitting each SAXS data as a Gaussian number distribution of hard spheres. Custom MATLAB R2014a programs were used for the analysis. Here, the scattering intensity, $I(q)$, for a single sphere of radius $R$ is
Eq. 1 \[ I(q) = \rho_0^2 \nu^2 \frac{9(\sin qR - qR \cos qR)^2}{(qR)^6} \]

where \( \rho_0 \) is the electron density contrast, and \( \nu \) is the volume of the sphere, respectively.

The total scattering curve was calculated as a sum across a Gaussian distribution, taking into account both the scattering strength and the relative abundance of each sphere radius sampled. The fitting was performed by minimizing the residuals on a \( \log(Iq^4) \) vs \( q \) basis. The data were well fit by modeling form factors alone.

Results and Discussion

With the knowledge that composition could be used as a tool to alter the resulting magnetic properties for application in MagMED, a series of substituted metal ferrites were synthesized and systematically characterized to investigate their properties in relation to MagMED. Particles were synthesized using a one pot thermal decomposition method outlined in the experimental section. Characterization techniques applied were energy dispersive X-ray spectroscopy (EDX), bright field transmission electron microscopy (TEM), AC calorimetry, and inductively coupled plasma optical emission spectroscopy (ICP-OES). SAR measurements on particles synthesized for this paper were done using a field of 20 kAm-1 at a frequency of 206 kHz.

Bright field imaging (figure 2) shows slight variation of size within samples containing the same dopant. This variation within a sample set is much smaller than the variation between samples with different dopant materials. This is to be expected as both lattice constants and kinetic growth are highly variable and depend on the chosen dopant.
Figure 2. Bright field TEM of substituted metal ferrites synthesized by one pot thermal decomposition of metal acetylacetonate precursors at $350^\circ C$. 
It is important to note that size control for each of the varying dopants must be independently developed as the kinetics and thermodynamics at play during the reaction vary greatly depending on the dopant metal chosen. Because nanoparticle volume plays a large role in defining optimal parameters for energy transfer efficiency, these differences in size do not allow for highly accurate comparison of for example nickel ferrite and cobalt ferrite. One can instead come to a set of broad conclusions evaluating how size and dopant loading may affect the SAR values of given materials.

Complex morphologies in particles with differing phase as well as chemical makeup in mixed ferrite systems are also possible, and seem to depend on the precursors chosen for the synthesis. This difference in morphology is illustrated in figure 3, where different metal acetylacetonates were chosen as the organometallic precursors for the thermal decomposition reactions.
Because metal ferrites were the target of investigation, the iron precursor was typically included in higher amounts than the metal dopant. Varying core shell morphologies may correspond to largely dissimilar decomposition temperatures between precursors. Cobalt (II) acetylacetonate has a similar decomposition temperature to the iron (III) acetylacetonate, and it shows good incorporation throughout the lattice and relatively accurate stoichiometry corresponding with the expected dopant concentration. Manganese (II) acetylacetonate however has a much different decomposition temperature, and core
shell/Janus effects are observed where local manganese loading is extremely high compared to the particle shell. Stoichiometry is also highly variable corresponding with the large differences in the decomposition. Thermal decomposition reactions are even further complicated as an intermediate organometallic compound is formed from the free metal ions, this is normally an oleate complex. The morphological effect due to precursor decomposition is highly important when looking at particle optimization for MagMED, as core shell or highly local loading will most definitely influence the materials’ properties including anisotropy and magnetic saturation. Decomposition differences in this secondary in-situ formed precursor are still under investigation. The variability in stoichiometry and dissimilarity in precursor decomposition temperature can also affect concentration measurements of MagMED contributing metal ions and skew SAR results.
Table 1. Intended and actual particle composition as measured by EDX elemental mapping

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intended</th>
<th>EDX Actual</th>
<th>ICP Actual</th>
<th>SAR (W/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe</td>
<td>M</td>
<td>O</td>
<td>Fe</td>
</tr>
<tr>
<td>Fe</td>
<td>2.75</td>
<td>0.25</td>
<td>4.00</td>
<td>2.6±0.16</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>0.50</td>
<td>4.00</td>
<td>2.6±0.16</td>
</tr>
<tr>
<td></td>
<td>2.37</td>
<td>0.63</td>
<td>4.00</td>
<td>2.5±0.25</td>
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<tr>
<td></td>
<td>2.25</td>
<td>0.75</td>
<td>4.00</td>
<td>2.3±0.20</td>
</tr>
<tr>
<td></td>
<td>2.125</td>
<td>0.875</td>
<td>4.00</td>
<td>2.2±0.49</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>1.00</td>
<td>4.00</td>
<td>2.1±0.21</td>
</tr>
<tr>
<td>Co$_2$Fe$_5$O$_4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.75</td>
<td>0.25</td>
<td>4.00</td>
<td>2.8±0.21</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>0.50</td>
<td>4.00</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.25</td>
<td>0.75</td>
<td>4.00</td>
<td>2.4±0.11</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>1.00</td>
<td>4.00</td>
<td>1.7±0.40</td>
</tr>
<tr>
<td>Mn$_2$Fe$_5$O$_4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.75</td>
<td>0.25</td>
<td>4.00</td>
<td>3.0±0.12</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>0.50</td>
<td>4.00</td>
<td>-</td>
</tr>
<tr>
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<td>2.25</td>
<td>0.75</td>
<td>4.00</td>
<td>2.9±0.29</td>
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<tr>
<td></td>
<td>2.00</td>
<td>1.00</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.75</td>
<td>0.25</td>
<td>4.00</td>
<td>2.4±0.32</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>0.50</td>
<td>4.00</td>
<td>2.5±0.16</td>
</tr>
<tr>
<td></td>
<td>2.25</td>
<td>0.75</td>
<td>4.00</td>
<td>2.2±0.19</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>1.00</td>
<td>4.00</td>
<td>2.0±0.067</td>
</tr>
</tbody>
</table>

$^{a)}$EDX maps done on FEI Titan 200kv S/TEM with an average of 50 particles analyzed.

The calculated SAR values shown in Table 1 illustrate the vastly different affect both composition and dopant concentration have on the energy transfer efficiency of a material. This is especially noticeable in the case of cobalt-substituted ferrite where a large peak in SAR (734.4 W/g) is seen at a very specific concentration. Interestingly, this peak corresponds with a large shift in peak loss frequency when examining the material using AC magnetometry (figure 4).
This indicates a shift in the effective anisotropy of the material to a higher value reaching a maximum at an optimal non-stoichiometric concentration of Co. This trend is also seen in the other doped ferrites including Mn where a peak in SAR value is seen at lower concentrations of the dopant material (679.2 W/g at [Mn] = 0.19) above which a large drop in SAR is observed (134 W/g at [Mn] = 0.56). It is obvious then that the optimization of these materials for MagMED should not be based on stoichiometric ferrites, but their substituted counterparts. It is also of note that the SAR values calculated of the metal ferrites were done in toluene and without surface modification. Both the medium chosen for SAR measurements as well as surface chemistry, especially large macromolecules such as poly(ethylene glycol), may very well have effects on the overall energy transfer efficiency of the material.
The increase in research of these complexly doped systems has given insight into maximizing the anisotropy of materials, including the partial doping of systems versus the stoichiometric equivalents.\textsuperscript{12} Although investigation of these complex materials is warranted, there are still disagreements in literature pertaining to anisotropy constants and optimum particles for less complex systems, such as magnetite or pure phase metal ferrites.\textsuperscript{13-18} Many of these differences in effective anisotropy can be attributed to differences in chosen field and frequency parameters or synthesis method resulting in variable phase purity, particle size dispersion, and clustering effects. This large amount of variety in chosen parameters is illustrated in Table 2 which shows a large number of values across the field space.
Table 2. Summary table of reported parameters related to MagMED

<table>
<thead>
<tr>
<th>Composition</th>
<th>Diameter [nm]</th>
<th>a)Synthesis</th>
<th>SAR [W/g]</th>
<th>Max Field [kA/m]</th>
<th>Max Freq. [kHz]</th>
<th>Sample Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$_3$O$_4$</td>
<td>8.3</td>
<td>TD</td>
<td>6.5</td>
<td>12</td>
<td>183</td>
<td>S.Toluene</td>
<td>12</td>
</tr>
<tr>
<td>Fe$_3$O$_4$</td>
<td>19</td>
<td>TD</td>
<td>2452</td>
<td>29</td>
<td>520</td>
<td>Aqueous</td>
<td>19</td>
</tr>
<tr>
<td>Fe$_3$O$_4$</td>
<td>30</td>
<td>TD</td>
<td>2614</td>
<td>.66</td>
<td>1000</td>
<td>Aqueous</td>
<td>20</td>
</tr>
<tr>
<td>Fe$_3$O$_4$</td>
<td>20</td>
<td>CP</td>
<td>1048</td>
<td>36.5</td>
<td>341</td>
<td>Aqueous</td>
<td>21</td>
</tr>
<tr>
<td>Fe$_3$O$_4$</td>
<td>20</td>
<td>CP</td>
<td>719</td>
<td>36.5</td>
<td>341</td>
<td>Solid</td>
<td>21</td>
</tr>
<tr>
<td>γ-Fe$_2$O$_3$</td>
<td>24</td>
<td>Pol</td>
<td>1992</td>
<td>21.5</td>
<td>700</td>
<td>Aqueous</td>
<td>22</td>
</tr>
<tr>
<td>Co$<em>{0.8}$Fe$</em>{2.4}$O$_4$</td>
<td>8.4</td>
<td>TD</td>
<td>40.4</td>
<td>12</td>
<td>183</td>
<td>S.Toluene</td>
<td>12</td>
</tr>
<tr>
<td>CoFe$_2$O$_4$</td>
<td>28</td>
<td>EC</td>
<td>133</td>
<td>40</td>
<td>101</td>
<td>Aqueous</td>
<td>23</td>
</tr>
<tr>
<td>Co$<em>{0.5}$Zn$</em>{0.5}$Fe$_2$O$_4$</td>
<td>19</td>
<td>CP</td>
<td>115</td>
<td>26.7</td>
<td>256</td>
<td>Aqueous</td>
<td>24</td>
</tr>
<tr>
<td>Mn$<em>{0.8}$Fe$</em>{2.2}$O$_4$</td>
<td>16.1</td>
<td>CP</td>
<td>103.4</td>
<td>2.3</td>
<td>1950</td>
<td>Aqueous</td>
<td>25</td>
</tr>
<tr>
<td>MnFe$_2$O$_4$</td>
<td>7.13</td>
<td>Pol</td>
<td>1.8</td>
<td>33.3</td>
<td>276</td>
<td>Aqueous</td>
<td>4</td>
</tr>
<tr>
<td>NiFe$_2$O$_4$</td>
<td>7.8</td>
<td>ST</td>
<td>189</td>
<td>25</td>
<td>765</td>
<td>S. DMSO</td>
<td>5</td>
</tr>
<tr>
<td>Ni$<em>{0.8}$Fe$</em>{2.2}$O$_4$</td>
<td>15.8</td>
<td>ST</td>
<td>326</td>
<td>25</td>
<td>765</td>
<td>S. DMSO</td>
<td>5</td>
</tr>
<tr>
<td>MgFe$_2$O$_4$</td>
<td>20</td>
<td>CB</td>
<td>85.6</td>
<td>26.7</td>
<td>265</td>
<td>Aqueous</td>
<td>1</td>
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<tr>
<td>Zn$<em>{0.4}$Fe$</em>{2.6}$O$_4$</td>
<td>15</td>
<td>TD</td>
<td>432</td>
<td>3.7</td>
<td>500</td>
<td>Aqueous</td>
<td>26</td>
</tr>
<tr>
<td>ZnFe$_2$O$_4$</td>
<td>8.4</td>
<td>Pol</td>
<td>28.19</td>
<td>33.3</td>
<td>276</td>
<td>Aqueous</td>
<td>4</td>
</tr>
</tbody>
</table>

a) TD=Thermal Decomposition, EC=Electro Chemical, SG=Sol-Gel, CP=Coprecipitation, CB=Combustion, ST=Solvothermal, Pol=Polyol Method. b) Many of the cited papers demonstrate SAR dependence on field and frequency. This chart only records the largest field and frequency used, or field reported with SAR values. c) An extended table is available in the appendix.

These vary widely in literature which is a problem, as it is difficult to accurately compare or evaluate materials for MagMED when major parameters such as field and frequency differ. Further aggregation of values reported in literature and standardization of these
parameters would be beneficial in parsing out materials best suited for clinical application. It is also important to understand the implications that particle synthesis has, and it may be of interest to derive the expected $K_{\text{eff}}$ value of a material for a given synthesis technique. This would both allow for easy comparison of newly prepared particle systems as well as comparison of a specific material between the multiple synthesis routes.

Focus on cobalt substituted Ferrites

From the initial one pot synthesis and characterization of the metal ferrites, the cobalt ferrite had the largest SAR value, as well as homogenous loading throughout the lattice of the substituted cobalt. This along with cobalt ferrites already high anisotropy led us to focus on the cobalt system. Traditionally, research in MagMED has been centered on the most commonly studied iron oxide phases, maghemite ($\gamma$-Fe$_2$O$_3$) and magnetite (Fe$_3$O$_4$). Despite significant research activity, there are currently limited commercial and clinical applications of these materials. Recently, to improve upon the material properties of iron oxides, researchers have begun to consider substituted and doped ferrites. These materials show a wide range of magnetic properties, including, tunable magnetic saturation, magnetocrystalline anisotropy, and blocking temperature. Coupled with recent advancements in synthesis, and increasing control over both size and morphology of nanoscale colloids, these new materials have been shown to exhibit properties that are greatly improved from those of Fe-ferrites. One of these select materials is cobalt ferrite (CoFe$_2$O$_4$), which has a much greater effective anisotropy due to the replacement of the Fe$^{2+}$ with the highly anisotropic Co$^{2+}$ ions. By maximizing the effective anisotropy
through Co doping, specifically between cobalt levels between 0.4 and 1 in $\text{Co}_x\text{Fe}_{3-x}\text{O}_4$, as well as identifying the optimal volume for energy release using a novel extended LaMer synthesis, the optimization of cobalt ferrite based materials for application in MagMED is possible.\textsuperscript{9,10} Herein, we describe a potential synthetic method to produce these materials in a size controllable manor, and the resulting properties of the particles relating to MagMED.

Extended LaMer Synthesis of Cobalt Ferrite

Metal oleate complexes used for the extended LaMer synthesis were produced via a truncated one-pot synthesis as is described by Vreeland et al. Initial data suggested that particle nucleation and formation began approximately 40 minutes into the reaction equating to 2 ml of 0.22 M precursor (44 µmols) with the first measurable SAR value occurring at 50 minutes when particle size was 10.8 nm (657 nm$^3$). Growth of particles continued up to 80 minutes, reaching a final diameter of 21.5 nm (5225 nm$^3$) with a sharp decrease in particle diameter to 14.8 nm (volume 1683 nm$^3$) at 85 minutes. This drop may correlate with the sudden decrease in the SAR value at the 85 minute time point as seen in figure 5.
Figure 5. Extended LaMer growth of Co$_{0.5}$Fe$_{2.5}$O$_4$ nanoparticles over time with particle volume determined by SAXS analysis compared to SAR values. Particle volume is linear up to 85 minutes. SAR shows no discernable trend. Linear best fit lines exclude the last point in the data shown.

SAXS analysis of this extended LaMer growth (Co$_{0.5}$Fe$_{2.5}$O$_4$) was consistent with linear volumetric growth with time of 160.8 nm$^3$/min with an $R^2$ value of 0.99 and a sudden decrease in nominal particle size at 85 minutes. The best-fit line indicated an induction time of 46 minutes prior to the start of growth, although this may not account for possible initial rapid catalytic growth via the Finke-Watzky mechanism.$^{27}$
Figure 6. SAXS data for the extended LaMer drip of Co0.5Fe2.5O4. A1 corresponds to 20 minutes into the reaction. Aliquots were taken every 5 minutes up until the final at 95 minutes. 

A) Intensity vs scattering vector (q) as the reaction progresses. B) Size distribution of particles at particular aliquots showing particle growth. Scattering data was first able to be fit at A6 showing small crystallites using a truncated gaussian. Subsequent aliquots show small dispersion until A14 where the distribution widens and shifts to a lower diameter.

The observed decrease in average particle size may occur as a result of reaching a critical size at which the nanoparticles precipitate from solution and the further subsequent addition of precursors results in secondary nucleation. This is of high importance as it shows that the extended LaMer synthesis for magnetite ported to a more complex ferrite system and needs to be fine-tuned to support each material on a case-by-case basis.
Although this instability was observed, SAXS and TEM data shows that Co$_{0.5}$Fe$_{2.5}$O$_4$ particles with diameters between 10 nm and 18 nm can be synthesized reliably using this method without major colloidal instabilities. SAXS data for the LaMer reaction shown in figure 6.

The instability of the particles at approximately 18 nm also leads to an interesting phenomenon where a secondary nucleation phase occurs. This is illustrated in figure 7 where we see the TEM images and the corresponding size analysis show a bimodal distribution with the primary distribution falling around the smaller (6 nm) diameter particles and a secondary distribution at a much larger size (17 nm). This may be in part due to the destabilization of particles happening at a fast enough rate that growth kinetics of particles still in solution cannot consume free metal monomer fast enough, allowing for a buildup in monomer concentration. This may lead to secondary nucleation event. Initial attempts at remedying this issue were made by lowering flow rate of the precursor injection. A 1 ml/hr flow rate caused issues with the synthesis itself as both the slow flow of N$_2$ gas and taking usable aliquots caused the reaction to “dry up” before meaningful data could be extracted. The 2 ml/hr flow rate produced particles that were inhomogeneous in both shape and size further exacerbating the issue with the inhomogeneity seen in the 3 ml/hr drip rate. It must be noted
Figure 7. TEM and the corresponding size analysis distribution of aliquots taken during extended LaMer reaction synthesizing Fe$_2$Co$_{0.5}$O$_4$. A) 80 minutes diameter=12.4 nm, standard deviation=1.1 nm. B) 100 minutes, diameter= 16.0 nm, standard deviation= 1.4 nm. C) 120 minutes diameter= 17.4 nm, standard deviation= 1.8 nm. D) 140 minutes, diameter small= 6.54 nm, standard deviation small= 2.3 nm, diameter large= 17.4 nm, standard deviation large= 1.2 nm. All scale bars are 50 nm.

that there are many additional variables to change including precursor concentration, capping ligand concentration, reaction temperature etc. and that exploration into the optimization of this synthesis is ongoing.
SAR vs. Particle Size

One of the many advantages in using an extended LaMer synthesis is that particle size can be controlled, and once a desired size is reached, the reaction can be quenched yielding a sample with relatively low dispersion and high uniformity. Alternatively, small aliquots can be taken throughout the reaction to isolate size as a lone variable. Expectedly as the reaction proceeds, the particles get larger and the SAR of the samples increases which can be seen in Figure 5. However, the SAR values calculated from the drip reaction aliquots were surprisingly low, with the highest at approximately 50 W/g.

It is important to note that the diameters given in this chapter (both TEM and SAXS) may not represent the true magnetic diameter. The particles may contain a relatively large magnetic dead layer which has been seen in iron oxide particles, which may explain the low SAR values exhibited by these particles. The low SAR values may also be artificially lowered due to the presence of unreacted precursor still left in the sample during measurement. Because of the constant drip of oleate precursor it is inevitable that some is taken up in each of the aliquots. Although chromatography was done to try and minimize this, it is difficult to fully separate all excess oleic acid and metal oleate from the particle sample. Any excess metal not incorporated into a particle would not contribute to the heating rate during calorimetry but would show up in the mass normalization, thus leading to an artificially low specific absorption rate. A longer drip reaction was run to confirm the SAR value limit as well as to see the effects of the secondary nucleation. As the reaction progressed an expected increase and unexpected sudden drop in SAR was seen, similar to previous reactions. What is interesting to note is
that a secondary increase and sudden drop in SAR was seen again after the first SAR increase and decrease cycle. This illustrates that the drip reaction under these conditions (Fe$_{2.5}$Co$_{0.5}$O$_4$ @ 3ml/min) showed an almost oscillatory effect through nucleation growth and destabilization seen in figure 8. These phenomena limit the usefulness in using this specific reaction with these parameters to synthesize large diameter particles but applications looking for size control of cobalt doped ferrites within the 10 to 20 nm regime may find the presented synthesis useful.

Investigation into different ligands, drip rates and precursor concentrations could potentially alleviate the secondary nucleation by changing the energetics allowing the particles to stay in solution, or change the metal monomer addition rates to eliminate secondary monomer buildup and nucleation.
Figure 8. Extended specific absorption rate as a function of reaction time showing oscillatory behavior from multiple nucleation, growth, and precipitation cycles.

Conclusions

Non stoichiometric metal ferrites have been synthesized via the one pot method and characterized via TEM, EDX, ICP and SAR to evaluate their usefulness in MagMED applications. EDX was of particular importance as it showed that morphology may play a large role in the determination of anisotropy and therefore heating rate. Cobalt doped samples showed high SAR values between Fe$_{2.5}$Co$_{0.5}$O$_4$ and Fe$_{2.4}$Co$_{0.6}$O$_4$. This material was investigated further using the extended LaMer drip synthesis to control size and evaluate it as a possible candidate for a high SAR material.

Size control up to approximately 20 nm was achieved using the Extended LaMer drip however a cyclic nucleation, growth, and destabilization was seen using both TEM and SAR values which limits the size. Further exploration into the experimental drip procedure must be done to optimize the synthesis as particle stability above 20 nm leading to secondary nucleation was observed. Initial experiments into changing the flow rate of precursor show promise but a more detailed exploration of drip rate, precursor concentration, and stabilizing ligand are necessary.
References


CHAPTER THREE

GLYCAN FUNCTIONAL MAGNETITE AS A NOVEL UNCONVENTIONAL ANTIBIOTIC

Contribution


This article is a shared first author publication between myself and Dr. Yash Raval. It is acknowledged in the article that equal work was done between the two individuals. My contribution to this manuscript was the design, synthesis, and characterization of the nanomaterials. This includes synthesis of the polymer, conjugation of the glycan, and all characterization of the material. I was also paramount in the design and application of the MagMED treatment of the bacterial cultures, as well as stability studies, and TEM of both the particles and the bacteria. The manuscript was also written as a joint effort with Dr. Yash Raval. I would like to extend a large acknowledgement and thank you to Dr. Yash Raval and Dr. Tzuen-Rong Jeremy Tzeng from the Clemson Microbiology Department. Without them the highly complex and collaborative nature of this work would not be possible. I would also like to thank Jamie Murbach, and Yves Cordeau from the Clemson Materials Science and Engineering Department for help in the intricate synthesis of some
of the materials required for this project. Lastly, I would like to thank my advisor Dr. O. Thompson Mefford, who was the guiding hand for all of the materials synthesis, characterization and application for the work I completed on this project.

Introduction

The emergence of anti-microbial resistance (AMR) bacteria has quickly taken hold as one of the greatest threats to modern medicine on a global scale, and the ramifications, if not dealt with in a timely manner, may be catastrophic. Currently, there are more than 160 different kinds of antibiotics available for therapeutic purposes. However, unrestricted and prolonged usage of antibiotics has resulted in rapid emergence of new strains of bacteria that have developed resistance to these drugs and, over time, evolved as multi-drug resistant microorganisms. Infections caused by such bacterial strains have resulted in prolonged hospital stays and an increase in outpatient costs and patient mortality and morbidity throughout the world. In a World Health Organization (WHO) global report on antimicrobial resistance, it is stated that AMR is a complex global public health challenge and that no single or simple strategy will contain the emergence and spread of AMR infectious organisms. A recent study by the US Center for Disease Control and Prevention estimates that every year, diseases caused by AMR strains of bacteria infect millions of people in the US, and thousands of them die annually due to lack of new antibiotics. The emergence of AMR also has an enormous socioeconomic impact. A 2014 report from RAND Europe, estimates the economic cost of AMR to be approximately 3.1% of global output gross domestic product (~2.4 trillion US dollars). Although recognized as an immediate issue, antibiotic discovery has declined with many major pharmaceutical
companies discontinuing their antibiotic development programs over the past decade due to low return on investment and difficulties in identifying new compounds. This reinforces the need for development of novel therapeutic approaches to address the AMR challenges.\textsuperscript{7-9}

Nanoparticles have been a highly investigated area as their properties differ from their bulk counterparts. Of these materials, iron oxide has found particular prevalence in the biomedical field with use in MRI contrast, drug delivery, cell separation, and cancer therapy.\textsuperscript{10-12} When an iron oxide particle is subjected to an alternating magnetic field, a hysteresis loop is completed in which the area relates to the energy release per cycle.\textsuperscript{13} By doing this at relatively high frequencies (kHz-MHz), the amount of energy transfer is great enough to affect surrounding cells.\textsuperscript{14} This phenomena was previously referred to as magnetic hyperthermia due to an observed temperature rise. However, recent studies have shown that cell death could be induced in the absence of bulk heating of the environment.\textsuperscript{14,15} In seeking a more descriptive term, we and others prefer to use the phrase magnetically mediated energy delivery (MagMED).\textsuperscript{16,17} The use of nanoparticles for biological applications requires stringent attention to surface chemistry as it affects reactivity, biodistribution, and stability.\textsuperscript{10,18} Much research has been put into optimizing surface chemistries for nanomaterials, with poly(ethylene oxide) (PEO) being one of the universally accepted coatings.\textsuperscript{19} PEO has been shown to prolong circulation time, greatly improve stability in protein and ion-rich environments, impart secondary functionality, and render stealth from the immune system.\textsuperscript{19} Further enhancement in stability may be imparted using a multi-anchored catechol as the iron binding moiety.\textsuperscript{18,20} This is highly necessary
as desorption of the polymer coating is an issue with many biomolecules including phosphonates and peptides, which have high affinity for iron oxide.\textsuperscript{21}

Though numerous groups have reported using alternate magnetic field along with magnetic nanoparticles as an alternative cancer therapy, limited work has been done in the same direction for treating bacterial infections.\textsuperscript{22-24} More importantly, to the best of the authors’ knowledge, no work exploring the use of nanoparticles for selective killing of targeted pathogen in mixed-bacterial culture settings has been reported. Attachment of bacterial pathogens onto the surface of mammalian cells is one of the foremost events in host-pathogen interactions. Several pathogenic bacteria are able to adhere to specific host-cell receptors via carbohydrate binding proteins, also called adhesins or lectins.\textsuperscript{25} These interactions are part of the signal cascade enabling bacteria to recognize the environment they are in and then turn on the cascade of processes leading to infections.\textsuperscript{26, 27} If these binding interactions are inhibited/interrupted then the chances of getting infection is greatly reduced.\textsuperscript{28}

The rapid advancement in the fields of nanotechnology and glycotechnology offers potentially new therapeutic options for treating bacterial infections. Over the last few years, few research groups have studied nanomaterials functionalized with multivalent carbohydrate groups and synthetic glycoconjugates for probing bacterial lectin-carbohydrate interactions.\textsuperscript{29, 30} Attaching these molecules onto the surface of nanomaterials has found numerous applications in pathogen detection/targeting,\textsuperscript{31} mammalian cell-receptor mimicking,\textsuperscript{32} drug delivery,\textsuperscript{33} and in anti-adhesion therapies.\textsuperscript{34} Specifically, carbon nanotubes,\textsuperscript{35, 36} gold nanoparticles,\textsuperscript{37, 38} diamond nanoparticles,\textsuperscript{39} polymeric
nanoparticles, and magnetic nanoparticles were reported to be bio-functionalized with different carbohydrate sugars/glycoconjugates and were utilized for studying lectin-carbohydrate interactions in different bacterial species. Given the excellent biocompatibility of using glycoconjugates molecules for functionalizing nanomaterials, not many studies have been carried out that can specifically detect and differentiate bacterial species in a mixed population both in vitro and in vivo.

*Escherichia coli (E. coli)* is one of the most common types of bacteria naturally occurring in the digestive tract of humans and animals. While most of the *E. coli* strains are harmless to humans and animals, there are few *E. coli* serogroups that are mainly responsible in causing infections. *E. coli* belonging to the enterotoxigenic (ETEC) group is responsible for causing traveler’s diarrhea in humans and bloody diarrhea in neonatal calves, pigs and lambs. The prevalence of the serogroups and presence of adhesins are considered to be the primary factors that facilitate intestinal colonization of ETEC. ETEC adhere to small intestinal microvilli membranes in vivo via adhesins that recognizes specific carbohydrate receptors and produce enterotoxins that act on enterocytes eventually causing diarrhea. Recently, numerous studies have reported an increase in multi-drug resistance of ETEC strains associated with the inclusion of growth-promoting antibiotics in animal feed. *E. coli* K99 (*EC* K99) is one of the commonly found ETEC strains in newborn farm animals responsible for causing colibacillosis. *EC* K99 has unique adhesins that can specifically recognize and attach to sialic-acid based glycolipid receptors present on the ileal villus epithelium of the small intestine. If these binding interactions are inhibited/interrupted, the chances of getting infection are greatly reduced. Our previous
studies have shown that nanoparticles functionalized with specific sialic-acid derivatives resulted in nanoparticles-induced aggregation of EC K99 along with excellent biocompatibility.\textsuperscript{37, 42}

In this study, it is hypothesized that multi-anchored magnetic nanoparticles conjugated with sialic-acid moieties that mimic host-cell receptors specific for EC K99 adhesins, would induce rapid clustering of EC K99 in the presence of these nanoparticles, and when such bacteria-nanoparticles aggregates are exposed to AMF, it would result in enhanced and selective inactivation/killing of EC K99. Our results demonstrate a clinically significant \~3-log reduction in CFU\textsuperscript{54} of EC K99 in the presence of GM3-MNPs used in conjunction with AMF. To our knowledge, it is for the first time that sialic-acid derived glycoconjugate functionalized magnetic nanoparticles have been employed for specific killing of target bacteria in the presence of AMF. This study serves as proof-of-concept that a high degree of selective bacterial killing can be obtained without using traditional antibiotics.

Experimental

Synthesis of Magnetite Nanoparticles

Magnetite nanoparticles were synthesized via thermal decomposition of an organometallic precursor in a high boiling point organic solvent.\textsuperscript{55} Iron (III) acetylacetonate (Alfa Aesar, 99\%) (1.074g) was combined in a 3-neck round-bottom with oleic acid (Alfa Aesar, 90\%) (15ml) serving as both the solvent and the stabilizing ligand. The vessel was initially purged with N\textsubscript{2} after which flow was adjusted to 0.1 L/min
ensuring an inert environment. The vessel was then heated to 350°C and left to react for 3 hours. At 3 hours, the reaction was quenched by removing it from heat, and left to cool under inert atmosphere. The resulting particles were dispersed in minimal hexanes and precipitated using a mixture of 3:1 ethanol (Fisher, Anhydrous) to acetone (Alfa Aesar, 99.5%) (x3). Particles were dispersed in toluene (VWR, 99.5%) and run through an organic based GPC column (Bio-rad S-X polystyrene beads) to further remove excess oleic acid ligand. TEM and size analysis was then done on the particles to ensure size specificity (figure 1).

Figure 1. Magnetite nanoparticles as synthesized before functionalization with PEO-PAA-dopamine polymer. A) Representative TEM image of the particles. B) Histogram depicting the particle size distribution.
Synthesis of Alkyne-PEO-PAA-Dopamine.\textsuperscript{20}

Poly(ethylene oxide) (PEO) synthesis: Ethylene oxide (Sigma Aldrich, 99.9%) distilled into a high pressure Parr reactor. Na-benzylphenone still dried tetrahydrofuran (THF, EMD Millipore, 99.9%) was injected along with a predetermined amount of an anionic initiator potassium bistrimethyl silyl amide (Sigma Aldrich, 1M in THF). The reaction was allowed to run for 72 hours and was subsequently terminated by opening the reactor to atmosphere. The synthesized PEO was precipitated with diethyl ether (VWR, 99.9%) and washed (3x) by dispersing it in chloroform, precipitating the polymer, centrifuging it at 15,000 RCF for 10 minutes and pouring off the residual supernatant. The PEO was then dried under vacuum overnight. HNMR was performed to calculate molecular weight as well as to confirm the presence of the protected amine end-group.

Under dry N\textsubscript{2} atmosphere, hetero-functional PEO and sodium hydride (Sigma Aldrich, 95%), in slight excess, were dissolved in dry THF. This was allowed to react for 30 minutes before an excess of propargyl bromide (Sigma Aldrich, 80% in toluene) was added drop-wise to the solution over 15 minutes. Once all of the propargyl bromide was added, the solution was allowed to stir for 12 hours at room temperature. The polymer was then purified by dissolution in chloroform and precipitation with diethyl ether (x3) and dried under vacuum for 12 hours. HNMR was performed to confirm the presence of an alkyne.

Deprotection of the trimethyl silyl group was done in 1M hydrochloric acid (VWR) in methanol (VWR, 99+%) and allowed to react for 4 hours. The polymer
methanol solution was diluted with DI water and the deprotected PEO was extracted (3x) with 50ml chloroform from which it was precipitated with diethyl ether and dried under vacuum. HNMR was performed to confirm the loss of the trimethylsilyl group.

Coupling of the PEO to the poly(acrylic acid) (PAA, Sigma Aldrich Mn=1,800) was done by dissolving both in dry N,N-dimethylformamide (DMF, Sigma Aldrich, 99.8%) in a 5:1 ratio. To this 1.1 excess (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, TCI, 98%) as well as catalytic amounts of 4-(dimethylamino)pyridine (DMAP, Alfa Aesar, 99+%) were added. The solution was allowed to stir for 12 hours. The solution was filtered, further purified by dissolution in chloroform following precipitation with diethyl ether (x3) and then dried under vacuum. HNMR was done to confirm PEO-PAA coupling.

Attachment of the anchor group: Dopamine hydrochloride (Alfa Aesar, 99%) was dissolved in DMF along with a 10% molar excess of triethylamine (Alfa Aesar, 99%) and allowed to stir for 30 minutes. In a separate round-bottom the PEO-PAA was dissolved in DMF along with EDC and catalytic amounts of DMAP. To this the dopamine hydrochloride solution was added and the combined solution was allowed to stir for 12 hours. The solution was then filtered, purified by dissolution in chloroform then precipitated in diethyl ether. The final product was dried under vacuum and analyzed to confirm the presence of the catechol.
Ligand exchange\textsuperscript{18}

Both magnetite nanoparticles as well as the PEO-PAA-dopamine were suspended separately in 5ml of chloroform. The particles at an approximate concentration of 3mg/ml of Fe and the polymer at approximately 40mg/ml. The polymer was then transferred to a scintillation vial capped with a septum and placed in a sonication bath. The bath was turned on and over the course of 15 minutes the magnetic nanoparticle solution was injected into the polymer solution. Once injection was finished, the combined solution was allowed to further sonicate for 15 minutes. The solution was then removed and put on a shaker table for 72 hours. The chloroform was then removed via rotary evaporator and further dried under vacuum. Deionized water (DI H\textsubscript{2}O) was then added and the vial was sonicated to help mediate suspension into the water. The water-based particles were then filtered through a 0.2-micron nylon filter to ensure large aggregates were not present. The solutions were then run through a GPC column (Bio-Rad P polyacrylamide beads) to separate excess polymer from the water dispersible particles.

Click Chemistry\textsuperscript{57}

The Cu(I) catalyzed Huisgen 1,3-dipolar cyclo-addition between the terminal polymer alkyne and the azido-GM3 was done in the aqueous phase with the azido-GM3 being the limiting reagent. A 2 mol % solution of Cu(II) sulfate (Sigma Aldrich, 99\%) was combined with equivalent molar amounts of the THPTA (synthesized according to Hong et al.) Cu chelating ligand and let to react for 10 minutes\textsuperscript{58}. This was then transferred into the aqueous alkyne-particle suspension and the azide-GM3 was then
added. After both additions a 10 mol% aqueous solution of (+)-sodium L- ascorbate (Sigma Aldrich 98+%) was added to facilitate the reduction of Cu(II) to Cu(I). The click reactions were left at room temperature for 12 hours, and were then purified using size exclusion chromatography.56

Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared (FTIR) spectroscopy microscopy was done using a Thermo-Nicolet Magna 550 FTIR spectrometer equipped with a Thermo-NicPlan FTIR microscope. 16 Scans were done for both the sample and the background. Samples were prepped by dropping a small amount of the water suspended sample on a germanium plate and left to dry under a heat lamp for 20 minutes. FTIR was done on the resulting films (figure 2).
Figure 2. FTIR spectra of particles before GM3 conjugation (A), GM3 molecule (B), and after conjugation (C). The lack of the azide peak in C at 2100 cm$^{-1}$ indicates purification of unbound GM3 after conjugation was successful.
Iron Concentration Determination

50 μl of magnetite suspension was dissolved in concentrated HCl, reduced and complexed with 1,10-phenanthroline (Sigma-Aldrich, 99%). UV-VIS was then performed to determine the amount of iron in the known volume. 59, 60

Transmission Electron Microscopy (TEM) of Bacterial Strains

Post AMF treatment of bacterial cells (MNPs concentration - 650 μg/ml), the mixture samples were removed from the glass vial and centrifuged at 7000 x g for 5 minutes. Later, the supernatant containing unbound MNPs was removed and the pellet was washed with 1x PBS in repetitive centrifugation cycles (3 times). After the final wash, the pellet containing MNPs and bacteria was fixed in cacodylate-buffered glutaraldehyde (3%, pH~7.2; Electron Microscopy Sciences, PA, USA) at 4°C for 12 hours. Subsequently, the sample was again washed in the cacodylate buffer thrice. 5 μl of the sample was dropped onto carbon-coated copper grid and was allowed to air dry for 4 hours. Finally, the sample was stained with 2% uranyl acetate (Electron Microscopy Sciences, PA, USA) solution for 15 seconds and blotted dry with filter paper.

AMF Treatment of Bacterial Strains in the presence of MNPs

As reported in our previous study, E. coli K99 (EC K99) was transformed with plasmid pGREEN (Carolina Biological Supply Company, NC, USA) carrying ampicillin-resistance marker gene. Avirulent E. coli O157:H7 strain B6914 (EC O157) was modified to be rifampicin resistant (100 μg/ml; TCI America, OR, USA) through
gradient-plate technique as previously described.  

EC K99 strain was routinely grown in tryptic soy broth/tryptic soy agar (TSB/TSA; EMD Millipore, MA, USA) supplemented with ampicillin (100 μg/ml; TCI America, OR, USA) and EC O157 strain was grown in tryptic soy broth/tryptic soy agar (TSB/TSA) supplemented with rifampicin (100 μg/ml).

For AMF treatment experiments, bacterial cultures were grown overnight under shaking conditions (250 rpm) at 37°C in TSB supplemented with appropriate antibiotics. Later, the bacterial cells were washed and centrifuged thrice in 1X sterile phosphate buffer saline (PBS). Approximately, 5 x 10^7 CFU of bacterial cells were suspended in 1X PBS based on optical density (OD_{600}) readings. Both the bacterial strains were mixed with different types of MNPs and at different concentrations of MNPs in a sterile microcentrifuge tube and this mixture was incubated at room temperature for 30 minutes under gentle shaking conditions to facilitate the binding interactions between bacterial adhesins and MNPs. At the end of incubation time-period, the mixtures of MNPs and bacteria were transferred to sterile glass vial. This vial was then placed in chamber of the alternate magnetic field generating instrument (EasyHeat Induction Heating System - Ameritherm®) that is covered with 5-turn induction coil, which was connected to polycarbonate recirculating water-bath for maintaining the sample temperature (37°C). A fiber-optic temperature probe (Neoptix™) was inserted inside this chamber to continuously monitor the temperature. The working conditions of the AMF instrument for the experiments were as follows: 480 Amps current, 207KHz frequency and magnetic field strength of 31 KA/m. The field was measured using an AC magnetic field probe (AMF Life Systems, Auburn Hills, MI). The vial containing the mixture of MNPs and
bacteria was then exposed to AMF treatment for different durations (30, 60, and 120 minutes). Different groups of control experiments were as follows: 1) mixture containing only bacterial strains suspended in 1X PBS; 2) mixture containing bacteria and MNPs but no AMF exposure. After AMF treatment, the above-mentioned mixture was serially diluted in 1X sterile PBS and 100 μl of sample from each dilution tube was spread-plated onto sterile TSA petri plates supplemented with appropriate antibiotics. Later, the TSA plates were incubated overnight at 37°C. Finally, the grown colonies on the TSA plates were counted and compared with control group plates and the reduction in colony counts was expressed as colony forming units per ml.

AMF Treatment of Mixed Bacterial Cultures in the presence of MNPs

To determine the targeted specificity of GM3-MNPs against EC K99 in mixed-culture conditions, both the strains of EC K99 (concentration - 5 x 10^7 CFU) and EC O157 (concentration - 5 x 10^7 CFU) were mixed in a single microcentrifuge tube. GM3-MNPs (650 μg/ml) were added to this mixture and the tube was incubated for 30 minutes at room temperature under gentle shaking conditions. Later, AMF treatment (time - 120 minutes) was applied to this tube as mentioned earlier. Control groups included 1) adding PEO-MNPs to mixture containing both strains of bacteria in the same tube and exposing/not exposing them to AMF; 2) adding GM3-MNPs to tube containing both bacterial strains but no AMF; 3) AMF exposure to tube containing only bacterial strains and no MNPs. Post-treatment, this mixture was serially diluted in 1X PBS and 100 μl of each dilution was spread plated in triplicates onto TSA plates supplemented with
appropriate antibiotics. The petri plates were incubated overnight at 37 °C and CFU reduction was compared to control group plates.

Bacterial Live/Dead Fluorescence Assay

To qualitatively determine the cell membrane integrity of bacterial cells, Live/Dead fluorescence assay were performed using BacLight™ Bacterial Viability Kit (L7007, Molecular Probes, Invitrogen, OR, USA). Freshly grown cells of EC K99 and EC O157 (5 x 10^7 CFU) in 1X PBS were mixed with PEO-MNPs and GM3-MNPs (Concentration - 650 μg/ml) separately in different microcentrifuge tubes. The mixture was allowed to incubate at room temperature for 30 minutes with gentle mixing after every 5 minutes. The tubes were then exposed to AMF therapy for 120 minutes. The samples were then prepared according to manufacturer’s protocol. Later, both fluorescent dyes i.e. SYTO 9 and Propidium Iodide were added to the samples. Finally, the samples were viewed under fluorescence microscope under different filters (SYTO 9 - Excitation/Emission - 485/510 nm; Propidium Iodide - Excitation/Emission - 485/630 nm) at 400X magnification. The images obtained under different filters were merged in ImageJ software (NIH, USA). Control group samples were not exposed to MNPs/AMF.

Microbial ATP Cell-Viability Assay

To measure the overall intracellular ATP levels of bacterial species (EC K99 and EC O157) before and after AMF treatment in the presence/absence of MNPs (concentration - 650 μg/ml, AMF treatment time - 120 minutes), microbial BacTiter-
Glo™ (Promega, Madison, WI) assay kit was used. The assay was performed according to manufacturer’s protocol with a small modification for samples containing MNPs. At the end of this assay, the plate was read in a micro-plate reader with luminescence capability (Synergy Hybrid H1, Biotek®) and the obtained results were expressed in relative luminescent units (RLUs).

Statistical Analysis

All the statistical analysis was performed using Graphpad Prism software (V 5.0, CA, USA). All the experiments were done in triplicates and data are expressed as Mean±SD. Statistically significant differences between the groups were evaluated by performing ANOVA. Post hoc group comparisons were calculated through Bonferroni post-tests. Results showing P values of ≤0.05, <0.01, and <0.001 were considered to be statistically significant.

Results and Discussion

Synthesis of GM3-MNPs

Magnetite nanoparticles were synthesized using a one-pot thermal decomposition of iron (III) acetylacetonate and oleic acid.59,63 Particles had an average diameter of 23.7 nm with a standard deviation of 1.55 nm (Figure 2). The moment vs. field (MvH) (Figure 3) measurement was done on the particles to confirm the super-paramagnetic behavior of the MNPs.
Figure 3. Moment vs. Field (MvH) loop showing the superparamagnetic behavior of the magnetite nanoparticles ($M_{sat} \sim 53 \text{ emu/g Fe}$).

Polymer design was based on work by Stone et al. where a multi-anchored binding approach showed increased stability in comparison to polymer ligands with a single binding moiety (Figure 4). In this work, however, the chosen catechol was not nitroDOPA but dopamine.
Figure 4. Left: 1) Anionic ring opening of ethylene oxide, 2) alkyne functionalization with propargyl bromide, 3) deprotection of primary amine, 4) coupling of PEO to PAA, 5) coupling of dopamine hydrochloride to the PEO-PAA. Right: Click reaction between polymer coated particles and GM3 molecule.

By using the multi-anchored approach, the stability in salt and protein buffer solutions is retained even when using dopamine as the anchoring group.\textsuperscript{18, 64} Hydrodynamic diameter and zeta potential of the PEO-MNPs before and after click-coupling of GM3 are reported in Table 1.
Table 1. Dynamic light scattering and zeta-potential measurements before and after GM3 conjugation.

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<th>Hydrodynamic Diameter Z Avg. (nm)</th>
<th>Zeta-potential (mV)</th>
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<tbody>
<tr>
<td>PEO-MNPs</td>
<td>78.8</td>
<td>-8.73</td>
</tr>
<tr>
<td>GM3-MNPs</td>
<td>88.8</td>
<td>-7.68</td>
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The specific absorption rate (SAR) value for the MNPs was measured to be 53.4 W/g (bulk temperature rise of solution from 37°C to 42°C), which is similar to the value for magnetite reported by Ma et al. The increase in the hydrodynamic diameter indicates the GM3 glycoconjugate was successfully coupled to the PEO-MNPs. Attenuated total reflectance Fourier transformed infrared spectroscopy (ATR-FTIR) also confirmed the GM3 coupling went to completion with the disappearance of the azide peak from GM3 at ~2110 cm⁻¹ and the appearance of a broad alcohol peak in the GM3-MNPs spectrum from 3620-3170 cm⁻¹ (Figure 2).

MagMED Inactivation of Bacteria

The efficacy of GM3-MNPs for specific inactivation of ETEC K99 via MagMED was compared to that of enterohaemorrhagic Escherichia coli (EHEC) O157:H7 strain since the two strains showed different receptor-binding specificities and EHEC O157:H7 strains are rarely harbored by pigs. Another E. coli strain ORN178 expressing mannose-binding type-1 fimbrial FimH adhesins was also evaluated and served as a negative control (data not shown). The MagMED inactivation of bacteria was assessed using a colony forming unit (CFU) reduction assay to determine the number of viable cells (CFUs) remained after treatment. The efficacy of AMF mediated killing of the target
bacteria, *EC K99*, in the presence of GM3-MNPs in a concentration- and time-dependent manner was evaluated. GM3-MNPs were mixed with *EC K99* and incubated at room temperature for 30 minutes to facilitate the binding between GM3 molecules present on the surface of GM3-MNPs and adhesin molecules of *EC K99*. PEO-MNPs were used as an internal control group to evaluate the role of the targeting moiety GM3. The magnetic field and frequency used in the AMF therapy remained constant for all treatment groups (31 kA/m and 207 kHz). Figure 5-A shows the final counts of CFU/ml of *EC K99* after treatment.
Figure 5. Colony Forming Unit (CFU) of E. coli strains after AMF treatment at different concentrations of MNPs and different time-intervals; A - CFU/ml of EC K99 after AMF treatment; B - CFU/ml of EC O157 after AMF treatment. Data is expressed as Mean ± SD (n=3); Statistical analysis – Analysis of Variance (ANOVA); * p-value <0.05, ** p-value <0.01, and *** p-value <0.001, where the population was compared to control population.)
The reduction in CFU/ml was found to be both time and MNPs concentration dependent. After 30 minutes of AMF treatment, ~1-log reduction in CFU/ml of EC K99 was observed at a particle concentration of 650 µg Fe/ml. After 60 minutes of AMF treatment, a significant ~2-log reduction of EC K99 \((p<0.01)\) was observed for both 280 µg Fe/ml and 650 µg Fe/ml concentrations of GM3-MNPs. Finally, at the end of 120 minutes of AMF treatment, an extremely significant ~3-log reduction in CFU/ml of EC K99 \((p < 0.001)\) was achieved with particle concentrations of 650 µg Fe/ml. Moreover, no significant killing of EC K99 was observed in experimental groups not exposed to AMF (Figure 6-A) indicating the non-toxicity of PEO-MNPs and GM3-MNPs to EC K99.
Figure 6. CFU/ml assay to determine viability of E. coli strains in the absence of AMF at different concentrations of MNPs and at different time-intervals: A - CFU/ml of EC K99 in the absence of AMF; B - CFU/ml of EC O157 in the absence of AMF. Data is expressed as Mean ± SD (n=3); Statistical analysis – Analysis of Variance (ANOVA).
Thus, targeted approach in AMF exposure can explain such effective reduction in CFU/ml of EC K99. Several research studies have shown similar results in reduction of bacterial population through antibody-targeted photo-inactivation process via near-infrared laser (NIR) in the presence of nanoparticles in both in vitro and in vivo settings.\textsuperscript{72-74} In our previous work, we have shown that sialic-acid sequences of GM3 molecule (Neu5Ac(α2-3)-Gal-(β1-4)Glcβ-sp) can specifically interact with S-type fimbrial proteins/adhesins present on the outer surface of EC K99 and induce rapid clustering of EC K99.\textsuperscript{37, 42} The presence of FanC, a major protein sub-unit present in S-type fimbriae, is primarily responsible for the specific attachment of EC K99 onto the ganglioside receptors, which are present on the host-cell surface.\textsuperscript{75} Therefore, it is likely that GM3-MNPs-induced bacterial aggregation plays a major role in the applied method.

Since GM3-MNPs are attached to or are in extremely near vicinity of EC K99, one can expect to see increased delivery of energy from the particles into the bacterial cells. Some reports have also suggested highly localized temperature increase taking place in the biological systems in the presence of MNPs when using AMF.\textsuperscript{76-78} For example, Huang \textit{et al.} functionalized the surface of MNPs with fluorophores that act as molecular temperature probes while remotely activating ion channels/neurons in the presence of AMF.\textsuperscript{79} Also, the observed drug release due to phase changes in the polymer near the surface of the particle suggests a local temperature increase.\textsuperscript{80} In these studies, too, the overall temperature of bulk particle suspensions remained constant or increased marginally. However, the exact mechanisms of how energy from the particle interferes with these pathways are still unknown and are in need of better characterization.
In the present study, the experimental group comprising of 140 µg Fe/ml that underwent AMF treatment did not show significant reduction in CFU/ml of *EC* K99 after 120 minutes of exposure. One possible explanation for this might have to do with the number of particles present in different concentrations of the GM3-MNPs. The lowest concentration group would have the least amount of MNPs. Thus, they might not be able to efficiently deliver the energy of the MNPs into the bacterial cells. PEO-MNPs at all concentrations and all time-points did not induce significant decrease in the colony counts of *EC* K99.

MagMED Inactivation of *EC* O157

Figure 5-B shows the overall reduction in CFU/ml of *EC* O157 after exposure to AMF in the presence of PEO-MNPs or GM3-MNPs. A ~1-log reduction in CFU/ml was observed with both PEO-MNPs and GM3-MNPs at particle concentration of 650 µg Fe/ml for 60 and 120 minutes. All other MNPs concentrations and time-points showed no significant reduction in colony counts of *EC* O157. No reduction in CFU/ml of *EC* O157 was observed in the absence of AMF (Figure 6-B) indicating the non-toxicity of PEO-MNPs and GM3-MNPs to *EC* O157. Several groups have observed certain degree of non-specific electrostatic interactions occurring between bacteria and nanoparticles when mixed with relatively higher concentrations of nanoparticles.\(^{81,82}\) Hence, it is possible that due to such non-specific interactions along with high MNPs-to-bacteria ratio, few MNPs might come in contact or close proximity with *EC* O157 resulting in delivery of some magnetic energy into these cells causing a limited ~1-log reduction in CFU.
Bacterial control groups exposed to AMF in the absence of MNPs showed no changes in the overall CFU/ml after 120 minutes of exposure indicating little to no effect of AMF itself on the cultures.

Recently, Nguyen et al. conducted a similar type of study wherein they showed that iron-oxide nanoparticles could rapidly induce biofilm dispersal in Pseudomonas aeruginosa (P. aeruginosa) through application of an alternating magnetic field. Their study showed rapid increase in the temperature of buffer solution when nanoparticles were mixed with them and exposed to magnetic field. Similarly, another study reported 4-log reduction in biofilm of P. aeruginosa when exposed AC magnetic field in the presence of iron-oxide nanoparticles. The authors have attributed this reduction in biofilm due to quick increase in bulk temperature of the system through magnetic hyperthermia. Thomas et al. used magnetic fluid hyperthermia in the presence of carboxylic-acid stabilized iron-oxide nanoparticles and achieved ~7-log reduction in population of Staphylococcus aureus. In all of the aforementioned studies, the killing/inactivation of bacterial species/biofilms was attained solely due to drastic increase in the bulk temperature of the working system through nanoparticles and hyperthermia. It would be worthwhile to note that the aforementioned studies were conducted in the presence of relatively high concentrations of MNPs (ranging from 1 mg/ml to 50 mg/ml) and higher instrument frequencies (e.g., up to 1.05 MHz). Using such high concentrations of MNPs in humans might cause serious concerns with regard to toxicity of MNPs. Also, none of the above listed studies utilized any kind of targeting moiety on nanoparticles for attaining specificity.
On the other end, this work demonstrates significant killing of \textit{EC} K99 at much lower concentrations (maximum concentration - 650 µg Fe/ml) and at relatively benign frequencies (i.e., 207 kHz). These results further support our hypothesis that the combination of magnetic fields and presence of GM3-MNPs are responsible for reduction in CFU/ml of \textit{EC} K99 via targeted AMF therapy. The reported CFU reduction within 120 minutes (i.e., 3-log in 2 hours) treatment compares favorably to conventional antibiotic treatments. For examples, Silva \textit{et al.} reported that ciprofloxacin at concentrations corresponding to 1x MIC (Minimal Inhibitory Concentration) reduced \textit{E. coli} population by 1-log over a 24-h study while at 2x and 4x times the MIC value, they observed a \n\n2.5-log reduction in the first 2 hours and a 4-log reduction after 24 hours of treatment.\footnote{In a similar study, Drago \textit{et al.} observed no reduction of \textit{E. coli} population at 1x MIC with levofloxacin or ciprofloxacin over a 24-h period while a 2-log and 3-log reduction at 4x MIC was observed 3 hours and 24 hours after treatment, respectively. It is conceivable that with fine-tuned MagMED treatment regimen, it is possible to improve the treatment outcomes of infections caused by multiple-drug resistant bacteria.}

As mentioned earlier, a moderate temperature increase (37°C to 42°C) was observed in the bulk particle suspension while measuring SAR values in the presence of magnetic field. To prove that the significant log-reduction of \textit{EC} K99 is not due to temperature increase alone, a CFU/ml assay on both the \textit{E. coli} strains at elevated temperature of 43°C was performed. The bacterial strains were incubated with PEO-MNPs and GM3-MNPs for 120 minutes and this mixture was kept inside the holding chamber of the AMF instrument by maintaining a constant temperature of 43°C for 120
minutes in the absence of magnetic field. No significant changes in CFU/ml of EC K99 and EC O157 were observed after exposing the bacteria to MNPs and at elevated temperatures for 120 minutes. This strongly suggests that inactivation of the bacteria cannot be attributed to temperature alone.

MagMED Inactivation of EC K99 and EC O157 in Mixed Culture

Pathogens interact with host in a heterogeneous environment; the efficacy of utilizing MagMED for killing of the target bacteria EC K99 was evaluated in a mixed-culture condition. Both strains of the E. coli cultures were mixed in the same container at equal concentrations and added either the GM3-MNPs or the PEO-MNPs at a maximum concentration of 650 µg Fe/ml. These mixtures underwent the same AMF exposure as described earlier for 120 minutes and the CFU/ml reduction assay was done to determine the inactivation rates of both strains of E. coli. As seen in Figure 7-A, a significant ~2.5-log reduction ($p<0.001$) in CFU/ml of EC K99 in the presence of GM3-MNPs and AMF exposure was observed. The other mixed culture containing PEO-MNPs did not show significant difference in CFU/ml numbers compared to controls in the absence/presence of AMF. In addition, ~1-log reduction ($p<0.05$) in CFU/ml of EC O157 was seen when the mixed-cultures were added with PEO-MNPs or GM3-MNPs and underwent AMF treatment for 120 minutes (Figure 7-B). This reduction in CFU/ml of EC O157 is comparable in both mixed-culture and pure-culture experiments. Compared to CFU/ml assay results of EC K99 in pure bacterial culture experiments where a ~3-log reduction
was observed, the results obtained from mixed-culture experiments showed a ~2.5-log reduction.

Figure 7. Colony Forming Unit (CFU) of E. coli strains in mixed-culture conditions exposed to AMF for 120 minutes in the presence of MNPs (650 µg Fe/ml); A - CFU/ml of EC K99 after AMF treatment; B - CFU/ml of EC O157 after AMF treatment. Data is expressed as Mean ± SD (n=3); Statistical analysis - 2-Way Analysis of Variance (ANOVA); * p-value <0.05, ** p-value <0.01, and *** p-value <0.001.
This minor difference could be the result of a change in nanoparticle-to-bacterium ratio in mixed-culture experiments. Since the nanoparticle-to-bacterium ratio was reduced by half under mixed-culture settings, the probability of GM3-MNPs interacting with \textit{EC} K99 as well as \textit{EC} O157 is also slightly reduced than in pure culture settings. To the best of our knowledge, this is the first report wherein killing of targeted bacterial strains via MagMED has been demonstrated in mixed-culture settings.

Investigating Interactions between GM3-MNPs and EC K99 via Transmission Electron Microscopy (TEM)

One of the many causes for inactivation/killing of bacterial cells could be due to physical damages of bacterial cell membrane that results from the presence of different types of nanoparticles.\textsuperscript{85, 86} In order to investigate the bacterial-nanoparticle interactions between GM3-MNPs and \textit{EC} K99, TEM analysis on the samples was performed before and after AMF exposure. Since the maximum reduction in CFU/ml of \textit{EC} K99 was achieved in the presence of 650 µg Fe/ml MNPs after 120 minutes, the same experimental conditions to investigate if AMF exposure can exert any specific morphological changes on the bacterial cell membrane of \textit{EC} K99 was followed. Visualizing TEM images, highly specific interactions taking place between GM3-MNPs and outer membrane of \textit{EC} K99 was observed. Figure 8-B shows the extent of GM3-MNPs specifically attached to \textit{EC} K99. The entire cell-surface of the bacteria was covered with GM3-MNPs before applying AMF. There was no visible morphological change seen on the bacterial cell surface of \textit{EC} K99, which could also indicate that the
nanoparticle system does not have an apparent inherent toxicity by itself towards bacteria. Also, GM3-MNPs induced bacterial aggregation of EC K99 was clearly visible in TEM images. In contrast, after applying AMF, the cell structure of EC K99 was seen to be extremely damaged as seen in Figure 8-D.

<table>
<thead>
<tr>
<th>E. coli</th>
<th>No AMF</th>
<th>With AMF</th>
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<tr>
<td></td>
<td>With PEO MNPs</td>
<td>With GM3 MNPs</td>
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<td>K99</td>
<td>A</td>
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<td>O157</td>
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Figure 8. TEM images of GM3-MNPs induced bacterial membrane damage of E. coli strains: A, B, E, F - Before AMF, and C, D, G, H - after AMF treatment for 120 minutes. Concentration of MNPs - 650 µg Fe/ml. Scale bar is 500 nm.

Please also note that the diameters of EC K99 were reduced to less than 0.5x0.25 µm from the typical 1x0.5 µm. The nanoparticles were found internalized in cells of EC K99 after rupturing the cell membrane. Concurrent findings were also reported in a few studies with regard to destruction of bacterial cell membrane via targeted photo-thermal
lysis in the presence of nanoparticles. Even after AMF exposure, GM3-MNPs were found to remain attached onto bacterial cell debris, which suggests strong binding interactions between GM3-MNPs and adhesin molecules of *EC K99* present on the bacterial cell surface. Interestingly, it was observed that both the polar ends of *EC K99* were found to be much more deformed and broken. However, not much damage was seen towards the horizontal length of *EC K99*. A large quantity of cell membrane debris was observed in the TEM analysis suggesting that *EC K99* would have undergone thermal lysis after AMF exposure possibly due to highly localized temperature increase.

Moreover, both PEO-MNPs and GM3-MNPs did not show any substantial attachment to *EC O157* cells (Figure 8E to 8H). Even the cell membrane morphologies looked similar to control group cells that were exposed to AMF without any MNPs. Thus, the TEM analysis and CFU/ml assays support our initial findings regarding the specific interactions occurring between GM3-MNPs and *EC K99* and that GM3-MNPs found on bacterial surface of *EC K99* play an important role in lysing the bacterial cells (bactericidal) when exposed to AMF.

**Bacterial Live/Dead Fluorescence Assay**

One of the common assays employed to check the membrane integrity of bacterial cells is to use propidium iodide dye. Since the TEM analysis showed extensive membrane damage of *EC K99* cells after AMF treatment, to further support these results, bacterial live/dead assays were performed. *E. coli* strains, after AMF exposure in the presence of MNPs, were stained using mixture of SYTO 9 and propidium iodide dyes.
SYTO 9 is a green-fluorescent dye that stains both live and dead bacterial cells by entering inside them. Conversely, propidium iodide can only permeate those bacterial cells whose cell membrane structures have been compromised and stains them red. For this assay, the bacterial strains were mixed with different types of MNPs.

<table>
<thead>
<tr>
<th>E. coli</th>
<th>No AMF</th>
<th>With AMF</th>
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<tr>
<td></td>
<td>With PEO MNPs</td>
<td>With GM3 MNPs</td>
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<td>K99</td>
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<td>O157</td>
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**Figure 9. Live/Dead Staining Assay using SYTO 9 and Propidium iodide dyes.** Both strains EC K99 and EC O157 are stained with mixture containing the above-mentioned dyes. The bacterial strains were initially mixed with PEO-MNPs and GM3-MNPs (concentration - 650 µg Fe/ml) and then exposed to AMF treatment for 120 minutes. Live bacterial cells appear green in color and dead cells appear red in color. All the images are merged together for both green and red channel filters of the microscope. A, B, E, and F represent E. coli cells incubated with MNPs but without AMF treatment. C, D, G, and H represent E. coli cells incubated with MNPs in the presence of AMF. Magnification - 400X, Scale bar - 100 µm.
Figure 9 shows the results obtained after performing live/dead-staining assays. As clearly seen in Figure 9-D, more than 95% of *EC K99* cells were stained red in color after exposure to AMF in the presence of GM3-MNPs. This proves that indeed *EC K99* cells experienced extensive cell membrane damage due to AMF. Also, ~50% of *EC O157* cells were stained red which suggests partial membrane damage (Figure 9G-9H). In contrast, both *E. coli* strains stained green in color in the presence of MNPs but without exposure to AMF (Figure 9 A, B, E, F). The control group of bacterial cells in the absence of both MNPs and AMF also stained green in color. These results further support the non-toxic nature of the nanoparticles. Several research groups obtained similar results for live/dead staining assay when magnetic nanoparticles were used as antibacterial agents.\(^{24, 82, 90}\) These results correlate with those obtained in CFU reduction assay wherein *EC K99* and *EC O157* showed ~3-log reduction and ~1-log reduction in CFU/ml, respectively, after 120 minutes of AMF treatment in the presence of GM3-MNPs.

ATP Assay

The amount of ATP level present in any cell determines its metabolic state. In the presence of toxic materials/chemicals, the metabolic state of the cell can change and the intracellular ATP levels could drop because of toxicity. Higher levels of intracellular ATP levels indicate that the cell is metabolically active and their levels directly correlate to the actual number of bacterial cells present in the solution. Currently, several antibiotics available in the market exert their effects on targeting bacterial membrane
components to eradicate infections\textsuperscript{91,92}. To further demonstrate and explain the effect of AMF exposure on the biochemical metabolism of the inner cell membrane, an ATP assay based on luminescence was conducted to evaluate final ATP levels of \textit{EC K99} and \textit{EC O157} after exposing them to AMF for 120 minutes in the presence of different types of MNPs at their highest concentrations (650 \textmu g Fe/ml).
Figure 10. Intracellular ATP levels of bacterial strains in the presence of MNPs using BacTiter Glo. A - ATP levels of EC K99 after 120 minutes of AMF treatment in the presence of MNPs; B - ATP levels of EC O157 after 120 minutes of AMF treatment in the presence of MNPs. Data expressed as Mean ± SD (n = 3); Statistical Analysis - 2-Way Analysis of Variance (ANOVA). * p-value <0.05, ** p-value <0.01, and *** p-value <0.001.
As seen in Figure 10-A, a substantial decrease in intracellular ATP levels of \textit{EC K99} ($p<0.001$) after 120 minutes of AMF treatment can be observed in the presence of GM3-MNPs only while \textit{EC K99} in the presence of PEO-MNPs with/without AMF did not show significant changes in ATP levels. In contrast, the intracellular levels of \textit{EC O157} were found to be slightly reduced both in the presence of PEO-MNPs or GM3-MNPs with AMF exposure (Figure 10-B). A similar decrease in ATP levels was reported in \textit{P. aeruginosa} when they were subjected to magnetic induction in the presence of iron-oxide nanoparticles.\cite{24} One possible explanation for reduced ATP levels in the presence of AMF exposure can be the decline in membrane potential of bacteria, which could lead to interruption in ATP synthesis mechanisms (e.g., reduced proton motive force), membrane depolarization and eventually cell-death.\cite{86,93,94}

It is worth noting that \textit{EC O157} when treated with AMF in the presence of PEO-MNPs or GM3-MNPs resulting in similar limited level of cell damage, reductions in viable cells and intracellular ATPs. These results indicated that the changes are independent of the GM3 functional groups present on the surface of MNPs. The susceptibility of \textit{EC O157} to localized temperature changes due to proximity could be due to a lower thermal decimal reduction time ($D$-value) of \textit{EC O157} than that of \textit{EC K99}.\cite{95}

Conclusion

Proof-of-concept multi-anchored glycoconjugate GM3-MNPs that have high affinity to adhesin of \textit{EC K99} were synthesized. The prepared nanoparticle system can
specifically interact with adhesin molecules of *EC K99* and cause agglutination through nanoparticle-bacteria complex. Applying AMF treatment to such complex caused significant reduction in viability of targeted bacteria *EC K99* in both pure-culture and mixed-cultures settings due to possible highly localized temperature increase. Exposure to such conditions resulting in compromised membrane integrity of *EC K99* as determined through TEM imaging and Live/Dead staining. Moreover, GM3-MNPs coupled with AMF resulted in significant decrease in the overall intra-cellular ATP levels of the bacterium. Hence, the unique multi-anchored nanoparticle system in the presence of AMF can be effectively used as novel non-antibiotic platform for local and selective inactivation of the target bacteria in biological systems without affecting the viability of nearby cells/tissues. In the event of gastro-intestinal (GI) tract infections caused by ETEC pathogens, administered antibiotics can disrupt/destroy beneficial gut micro-flora in addition to pathogens. It could cause various side effects in the human body along with giving rise to antibiotic-resistant bacterial strains. The presented system can find useful applications in treating such infections in animals and humans and in conditions when administered antibiotics, especially those of the last-line-of-defense drugs, fail to eradicate the infections due to drug-resistance. Future studies will involve optimization of particle parameters including nanoparticle core-size and polymer coatings, as well as a detailed investigation on the effects of field strength and frequency to maximize killing rate of clinically relevant multi-drug resistant bacterial pathogens. Additionally, biocompatibility of our nanoparticle system will be evaluated in human cell-lines and small animal models.
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CHAPTER FOUR
HEPARIN FUNCTIONAL MAGNETITE FOR TREATMENT OF NEOINTIMAL HYPERPLASIA

Contribution

This chapter is adapted from the article; In vitro studies of heparin-coated magnetic nanoparticles for use in the treatment of neointimal hyperplasia, Nanomedicine: Nanotechnology, Biology and Medicine, Benjamin D. Fellows, Nardine Ghobrial, Elliot Mappus, Andrew Hargett, Mark Bolding, Delphine Dean, Olin Thompson Mefford, Volume 14, Issue 4, 2018, Pages 1191-1200.¹

My main contribution to this project was the synthetic design of the particles. This includes the nanomaterial synthesis, surface chemistry and functionalization. I was also a contributor in the discussion and interpretation of the results and the primary author on the manuscript. I would like to extend a special acknowledgement to Nardine Ghobrial, Elliot Mappus, Andrew Hargett, and Dr. Delphine Dean. They conducted the biological assays to obtain the relevant cell data. Without them this research would not be possible. I would also like to mention another collaborator, Dr. Mark Bolding, who was kind enough to do animal model MRI with our materials.

Introduction

Currently cardiovascular disease (CVD) is the leading cause of mortality worldwide where it accounts for approximately 30% of all deaths.² Cardiovascular disease (CVD) continues to grow in prevalence and overall healthcare burden, coupled with an
increasing elderly population within the United States, estimated total US healthcare cost due to CVD is projected to exceed $1 trillion by 2030.\textsuperscript{3} Within CVD, atherosclerotic cardiovascular disease (ACD) has become an epidemic that will only get worse with the lowering of communicable disease related deaths, urbanization of third world countries and the increasing longevity of the a populations lifespan.\textsuperscript{4} In ACD, affected arteries are occluded by a build-up of plaque within the vessel lumen. Over time, this limits blood flow, potentially causing downstream problems such as heart attack, stroke, and in many cases death. To prevent this resulting morbidity, many patients eventually require intervention to remove or reduce the blockage. For such cases, percutaneous coronary intervention (PCI) has become a standard procedure, utilizing stent and/or balloon angioplasty to increase lumen diameters to satisfactory levels.\textsuperscript{5, 6} While highly effective in immediate improvements to the lumen diameter, these procedures may suffer from complications, particularly by restenosis. When stents are used, restenosis often occurs by neointimal hyperplasia, an excessive proliferation of vascular smooth muscle cells within the vessel, occluding the vessel once again.\textsuperscript{7-10}

Current treatment options for neointimal hyperplasia focus on prevention, namely by drug-eluting stents (DES).\textsuperscript{11, 12} This treatment has been largely effective in reducing overall restenosis rates. DES reduced the incidence of in-stent restenosis (ISR) from 20-40\% with bare metal stents to 3-20\% with DES.\textsuperscript{13} While DES have reduced the rate of restenosis dramatically, they have introduced new complications such as late stent thrombosis due to delayed endothelialization.\textsuperscript{14-17} Endothelial cells in the tunica intima act as a selectively permeable barrier to prevent contact between VSMCs and growth factors.
circulating in the blood. Consequently, the endothelial layer inhibits intimal hyperplasia.\textsuperscript{18} Most currently available DES use antiproliferative drugs such as paclitaxel and sirolimus, which reduce the proliferation in both smooth muscle cells and endothelial cells, leading to delayed endothelialization. DES also have limited control over drug payload, dosage, or reloading.\textsuperscript{19, 20}

For optimal performance, this therapy option should be effective in cases where DESs fail and offer localized treatment without systemic effects. In addition to its widely known anti-coagulation effects, heparin has been shown in literature to inhibit VSMC proliferation after endothelial injury.\textsuperscript{21-26} It has also been shown to promote re-endothelialization, which accelerates the healing process.\textsuperscript{21, 23, 24, 27} One treatment option is systemically administering heparin. This treatment is associated with high rates of bleeding and other vascular complications due to the activation of antithrombin III leading to deactivation of thrombin and other proteases involved in blood clotting.\textsuperscript{28, 29} Recent advancements in magnetic targeting and magnetizable stents have shown the efficacy in localization of the desired payload.\textsuperscript{30, 31} Adamo et al. have reported an approximate 36\% dose retention of magnetically labeled endothelial cells on a magnetized stent compared to 0.7\% retention without a field present.\textsuperscript{32} Polyak et al. have also shown the effectiveness of this magnetic cell therapy using magnetically labeled endothelial cells to prevent lumen vessel narrowing.\textsuperscript{33} Other novel therapeutics have used this strategy to deliver high payload percentages to stent areas, including delivery of vectors for gene therapy.\textsuperscript{34} Herein, we describe the synthesis and characterization of a combined magnetic targeting carrier system paired with a pharmaceutical agent (heparin) to accomplish highly localized treatment
without full body circulation. The heparin-coated magnetite nanoparticles discussed in this manuscript could utilize this mechanism deliver localized anti-coagulation and antiproliferative effects at low concentrations effectively serving as a low toxicity targeted therapeutic for neointimal hyperplasia.\textsuperscript{35}

Experimental

Iron oxide nanoparticle synthesis

See chapter 3.

Polymer Synthesis

Poly (ethylene oxide), PEO synthesis: PEO was produced via previously reported methods.\textsuperscript{36} Briefly, ethylene oxide (Praxair, >99\%) was distilled into a high pressure Parr reactor. Dry tetrahydrofuran (BDH, 99\%) (THF) was injected along with a predetermined amount of an anionic initiator, in this case potassium bistrimethyl silyl amide (Sigma Aldrich, 0.5M in toluene). The reaction was allowed to run for 72 hours and was subsequently terminated by opening the reactor to atmosphere. The synthesized PEO was precipitated with diethyl ether (BDH, 99\%) and washed (3x) by dispersing it in chloroform (BDH, 99.8\%), precipitating the polymer, centrifuging it and pouring off the residual supernatant. The PEO was then dried under vacuum overnight. HNMR was performed to calculate molecular weight as well as to confirm the presence of the protected amine endgroup. Deprotection of the trimethyl silyl group was done in 1M hydrochloric acid
(BDH, 37%) in methanol (BDH, 99.8%) and allowed to react for 4 hours. The polymer methanol solution was diluted with water and the deprotected PEO was extracted (3x) with chloroform from which it was precipitated with diethyl ether and dried under vacuum. HNMR was performed to confirm the loss of the tri methyl silyl group.

Coupling of the PEO to the poly(acrylic acid) (Sigma Aldrich, 1,800 g/mol) was done by dissolving both in dry THF (BDH, 99%) in a 5:1 ratio in the presence of a 1.1 molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (TCI, 98%) (EDC). The solution was allowed to stir for 12 hours. The solution was filtered, further purified by dissolution in chloroform following precipitation with diethyl ether (x3) and then dried under vacuum. HNMR was done to confirm PAA-PEO coupling. Attachment of the anchor group: Dopamine hydrochloride (Sigma Aldrich, 98%) was dissolved in dimethylformamide (Acros Organics, 99.8) (DMF) along with 1.1 excess triethylamine (Fisher Scientific, 99%) and allowed to stir for 30 minutes. In a separate round bottom flask the PEO-PAA was dissolved in DMF along with a 1.1 molar excess of EDC. To this the dopamine hydrochloride solution was added and the combined solution was allowed to stir for 12 hours. The solution was then filtered, purified by dissolution in chloroform then precipitated in diethyl ether. The final product was dried under vacuum and analyzed via HNMR to confirm the presence of the catechol.

Ligand exchange

See chapter 3.
Modification of polymer coated iron oxide

Poly(ethylene oxide) (PEO) coated iron oxide nanoparticles (PCIO) were further modified to have pendant primary amine groups for (3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) coupling of heparine instead of the hydroxyl moieties present after ligand exchange. Particles were dispersed in dimethylformamide (DMF), and reacted with an excess of 3-chloropropylamine (Sigma Aldrich, 98%) in the presence of heavy excess triethylamine. Triethylamine was added to stay any adverse effects from the resulting acid from the EDC salt. After 8 hours, the particles were precipitated with diethylether, dispersed in water and run through a gel permeation chromatography (GPC) column (BIO-RAD, Biogel P-100 gel) to purify off excess reactants.

Iron oxide-heparin conjugation: Amine terminated polymer coated iron oxide particles (NH$_2$-PCIOs) were suspended in deionized (DI) water for heparin conjugation. The molar amount of heparin (MP Biomedical, sodium salt low molecular weight) used in the conjugation was approximately a 3:1 molar ratio of heparin to particle bound primary amine. A 1.2 molar excess of both N-hydroxysulfosuccinimide (Thermo Scientific) (sulfoNHS) and EDC were added to the aqueous heparin solution and allowed to stir for 30 minutes. The heparin-sulfoNHS complex was then combined with the particle solution and left to react for 3-5 hours. Purification of excess heparin was done using GPC (BIO-RAD, Biogel P-100 gel). Samples of the eluent were collected after the particle band to confirm separation of unbound heparin.
Quantification of surface bound heparin

1,9-dimethylmethylene blue (DMMB) assays were used to quantify the amount of heparin attached on the nanoparticles and confirm the success of heparin attachment. Using a round-bottom 96 well plate, 180 µL of DMMB solution was added to 20 µL of sample. Standard curves 0-100 µg/mL of heparin and chondroitin sulfate (Sigma) were found to not be significantly different. Samples were agitated for 5 seconds, and the absorbance at 530 nm was immediately read using a microplate reader. The coating of heparin/nanoparticle was determined using the measured DMMB absorbance, the linear regression equation, and the concentration of nanoparticle from the iron determination assay.

Conjugated heparin and particles were purified from excess unbound heparin via size exclusion chromatography. Visually the particles show up as a dark brown band on the column. Fractions were taken to look at the heparin concentration vs. elution volume. The initial spike which contained the particles shows a sharp increase and then decrease in heparin followed by a sharp increase when the free heparin was eluted.

Dynamic light scattering and zeta potential

Dynamic light scattering (DLS) and zeta potential (ZP) were performed on a Malvern Nano ZS zetasizer using a 633 nm laser. Both DLS and ZP were run using particle samples suspended in DI water containing a small amount of electrolyte (NaCl). All reported hydrodynamic diameters reflect the intensity weighted average.
The DLS determined hydrodynamic diameter shows an increase in the diameter after modification with polymer to 150 nm. Not much change is seen with further modification of heparin the end group or heparin. Heparin was determined to be attached via DMBB analysis of the purified particles.

Transmission electron microscopy

To assess the cell uptake of the nanoparticles, transmission electron microscopy (TEM) was used. Cells were cultured in standard incubation conditions using standard cell culture media (CCM). Once the cells were 90% confluent, nanoparticles were added at a concentration of 100 µg/mL diluted in CCM. After a 4-day incubation period, the cells were trypsinized and centrifuged to form a pellet. The pellet was fixed with 3% glutaraldehyde, osmified using 1% osmium tetroxide in water, dehydrated in a series of ethanol, and then infiltrated and embedded in catalyzed medium grade LR White embedding medium [Electron Microscopy Sciences/ 14380]. Ultrathin sections (70-90 nm) were cut using a microtome and mounted on Formvar coated copper grids [Electron Microscopy Sciences/FCF200-Cu]. A Hitachi Transmission Electron Microscope (TEM) H7600 was used for imaging. Energy Dispersive X-Ray Analysis (EDAX) was used to confirm the presence of iron inside the cells.

Particle characterization to show homogeneity and relative size using TEM. Particles were expected to be unblocked due to the size of the core. This is important for future application in magnetic targeting to magnetizable stents.
Iron concentration assay

Iron concentrations were determined using a modified version of ASTM standard E 394. A small sample of iron oxide particles was dissolved using approximately 200 µl of concentrated HCl. The resulting solution is diluted to 10 ml and 5 stock solutions containing 0.5, 1, 1.5, 2, and 2.5 ml of the original stock are made. Fe is reduced using hydroxylamine hydrochloride (Alfa Aesar, 99%), complexed with 1,10-phenanthroline (Sigma Aldrich, 99%), titrated via sodium acetate (Sigma Aldrich, 99%)-acetic acid (Mallinckrodt Chemicals, 99.7%) solution, and finally diluted to 10ml. This phenanthroline complex has high visible spectrum absorption and can be used to measure iron content by creating a standard curve and comparing unknown sample absorbance to the standard curve.

Vibrating sample magnetometry

Hysteresis loops of the iron oxide nanoparticles were done at the University of South Carolina using a vibrating sample magnetometer (VSM) attachment on a Quantum Designs physical property measurement system. The M vs. H loops were done at 300K using a field sweeping ±15,000 Oe. The nanoparticle sample was dripped on a cotton q-tip, fully dried, and placed under vacuum for measurement. The sample q-tip was digested by heating to 600 °C and the remaining iron was assayed. Subtraction of the paramagnetic of the q tip was done in post processing of the data.

Magnetization curve showing the superparamagnetic character of the heparin loaded nanoparticles at room 300 Kelvin. Saturation magnetization is in the expected range
for magnetite. This also illustrates the oxidative stability after being transferred into aqueous solution as we do not see a drop in expected magnetic properties.

Magnetic resonance imaging

All animal procedures were approved by the Institutional Animal Care and Use Committee of University of Alabama Birmingham (UAB) and adhered to the experimental animal care guidelines. Female athymic nude mice, 6-8 weeks of age were obtained from Charles River Breeding Laboratories and maintained under controlled conditions (25 °C, 65% humid, and 12 h light/dark cycle) at the UAB animal facility. Mice were anesthetized with isoflurane (2-3%) and placed in a custom-made mouse bed equipped with an anesthesia nose cone and a water bath circulation system to maintain the animal’s body temperature during imaging. A pneumatic pillow sensor was placed under the mouse’s chest and connected through an ERT Control/Gating Module (SA Instruments) to acquire the mouse’s respiratory cycle. The animal was then positioned inside the bore of a 9.4T Bruker BioSpec scanner (Bruker BioSpin, Billerica, MA) and MR imaged with a custom volume coil. T1- and T2- weighted images were collected with the following parameters: coronal slices, slice thickness 1.5 mm, voxel size of 0.2x0.2x1.5 mm voxels, 0.5 mm gap, FOV = 85×35 mm.

In vitro studies

Control conditions were created using untreated cells, heparin of molecular weight 3,000 g/mol solutions, and amine terminated nanoparticles (NP-NH2). Experimental
conditions were evaluated for rat vascular smooth muscle cells (VSMCs), porcine endothelial cells (PECs), and rat 3T3 fibroblasts (3T3s) between passages 4-9 and 70-90% confluence. All cell culture media, including control media, experimental media, and heparin solution solvent, was created as complete cell media (CCM) containing High Glucose Dulbecco’s Modified Eagle’s Medium, 10% fetal bovine serum, and 1% penicillin/streptomycin. All incubation conditions were conducted at 37°C and 5% CO2. For experimental conditions, both sets of nanoparticles were used in concentrations of 1, 10, 25, 50, and 100 ug/mL. Heparin-coated nanoparticles used in the in vitro studies were found to average 2.956 ug heparin per ug nanoparticle. Heparin solutions in CCM were used in concentrations of 1.56, 6.25, 25, 100, 400, and 1600 ug/mL.

Cell proliferation and metabolism

Cell proliferation and metabolic activity were assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay MTS kit by Promega. This kit uses a one-step colorimetric method to quantify viable cells. These viable cells reduce the MTS tetrazolium compound into a colored formazan product. Cells were seeded at 2,000 cells/well in CCM into a 96-well plate and incubated for 24 hours. Experimental conditions consisting of the full spectrum of nanoparticles, heparin solutions, and controls were applied and incubated for 48 hours. Following experimental conditions, cells were further incubated for 2 hours in a 1:5 volume ratio of MTS Assay kit and CCM. Absorbance was read at 490 nm using a microplate reader normalized against cell-free plates containing only CCM and MTS to compensate for any color distortion caused by media.
Live/dead assay

For this assay, cells were seeded at 20,000 cells/well into a 24-well plate and allowed 72 hours to adhere and grow in the standard CCM. Following this 72-hour incubation period, cells were checked to confirm confluence. For control conditions and analysis, control wells without nanoparticles were incubated in fresh CCM. Cells were then incubated under standard incubation conditions for a further 48 hours. The Live/Dead Assay Viability/Cytotoxicity kit (Life Technologies) was used to create solutions of 2 μM calcein acetoxyxymethyl (2 μM C), 4 μM ethidium homodimer-1 (4 μM Etd-1), and 2 μM calcein acetoxyxymethyl with 4 μM ethidium homodimer-1 (2,4 μM C+Etd-1) solutions diluted in phosphate-buffered saline (PBS) and protected from light. All experimental conditions were treated with 2,4 μM C+Etd-1 solution. Positive controls were treated with all three solutions and negative controls, created with 70% ethanol, were treated with 2 μM C and 4 μM Etd-1 solutions. All treated cells were incubated with live/dead assay kit solutions for 30 minutes and imaged using an EVOS fluorescent microscope for red/green fluorescence. This was additionally quantified using a microplate reader for fluorescence from 485 nm excitation to 530 nm emission and 530 nm excitation to 645nm emission.

Immunofluorescence staining

Cells were seeded at 2,000 cells/well into a 96 well plate and allowed to grow for 24 hours in CCM. This media was then replaced for experimental conditions. Control groups consisted of cells in CCM. Cells were incubated in experimental conditions for 48 hours and then fixed with 4% paraformaldehyde (Sigma Aldrich). VSMCs were targeted
for smooth muscle actin using Rabbit Polyclonal Primary Antibody to Alpha Smooth Muscle Actin (Abcam) paired with Secondary Antibody Donkey Anti-Rabbit (Abcam) to yield a green fluorescence for actin fibers. Endothelial cells were targeted for VE-Cadherin using Mouse Monoclonal Primary Antibody to VE-Cadherin (Santa Cruz Biotechnology) paired with Secondary Antibody Donkey Anti-Mouse (Abcam) to yield a red fluorescence on cellular junctions. 3T3 fibroblasts were targeted for collagen type I using Mouse Monoclonal Primary Antibody to Collagen Type I (Abcam) paired with Secondary Antibody Donkey Anti-Mouse (Abcam) to yield a green fluorescence on collagen. All cell types were further stained with (4’,6-diamidino-2-phenylindole) (DAPI) for cell nucleus. Following stain, cells were imaged using an EVOS FL Imaging System.

Statistical analysis

A 2-tailed student t test was used to preform statistical analysis. P values of $< 0.05$ are considered statistically significant.

Results and Discussion

There are a multitude of ways in literature that are used to synthesize magnetic nanoparticles with two of the most popular techniques being coprecipitation of iron salts and thermal decomposition of iron containing organometallics.\textsuperscript{37} For this study particles synthesized via a thermal decomposition method were used as core size and morphology are controllable and samples have a low size dispersion.\textsuperscript{38} Because of this, magnetic parameters can be controlled to a high degree due to low core size variation. The particles
as synthesized however are hydrophobic due to the stabilizing oleic acid ligands used in the high temperature synthesis. To remedy this, many groups have done work and used ligand exchange to confer stability in aqueous media.\textsuperscript{39-41} In this case a PEG-PAA-dopamine polymer complex was used for the ligand with PEG being the bio-inert stabilizing ligand and the catechol being the iron-binding moiety seen in figure 1.

Figure 1. Representative image of the heparin particle system and surface chemistry used for functionalization.

Dynamic light scattering (DLS) and zeta-potential studies were done on the particles before and after modification to ensure stability and investigate final hydrodynamic diameter of the particles in DI water.

Iron oxide core diameters were found to be approximately 24.3 ± 1.9 nm by transmission electron microscopy (TEM) via size analysis using Image J. This places the
particles firmly in the superparamagnetic regime, which was confirmed by the vibrating sample magnetometry showing no remnant magnetization and a saturation magnetization of approximately 62 emu/g Fe at 300 K. This is comparable to the particles used by Vosen et al. who showed particles with saturation magnetization of 62 emu/g and 92 emu/g could be used for magnetic targeting.\textsuperscript{34} It is also much greater than the particles used (~10 emu/g particles) for successful endothelial capture by Polyak et al. who also talk extensively about the stent magnetics and states that even far away from the gradient source the particles are magnetically saturated \textit{in vivo}.\textsuperscript{35} The size also indicates that issues with rapid renal clearance will not be encountered. The magnetic characteristics are important; as the particles will be used for magnetic adherence to magnetizable stents in future work.\textsuperscript{35, 42, 43}

The use of poly(ethylene glycol) (PEG) as a stabilizing ligand has been heavily used in biomedical applications, as it confers a high degree of particle stability in aqueous environments, renders the attached moiety/particle bioinert, and does not interact with the large amount of proteins and salts in bodily fluids. In this case a 5,000 g/mol molecular weight PEG brush was used lending more than enough steric interaction between particles confer stability in aqueous environments.\textsuperscript{41} Moreover, disassociation of the polymer brush from the particle surface is unlikely as the binding moiety to the particle consists of multiple catechols which have an enormous affinity for iron oxides.\textsuperscript{44} To create primary amine groups on the particle surface for EDC chemistry the polymer coated particles were reacted with chloropropylamine. Heparin loading of the particles was done using simple EDC sulfoNHS chemistry with the purification of unbound heparin being done by size exclusion chromatography (SEC) which is shown in figure 2.
Figure 2. DMMB assay on elution fractions from aqueous GPC purification of particle bound heparin and free heparin. Initial spike between 10 and 50 minutes corresponds to the particles which were seen in the column as a dark brown band.

The initial peak corresponds to visual elution of the heparin coated iron oxide particles, which were seen as a dark black/brown band in the column. The particle fraction collected was then tested via dimethylmethylene blue Assay (DMMB) and concentration was calculated to assess heparin loading per µg of iron.

The proliferative properties of the functionalized nanoparticles (amine and heparin) were investigated using three relevant cell lines in conjunction with both cell culture media as a negative control and raw heparin solution as a positive control. Cell viability and
proliferation were compared to the negative control. The three cell lines of interest included vascular smooth muscle cells (VSMCs), 3T3 fibroblasts, and porcine endothelial cells. These lines were chosen for their role in neointimal hyperplasia. MTS and live/dead assays were used to evaluate cell viability and proliferation. Comparisons between MTS and live/dead assays were used to determine if the reduction in cell counts were due to cell death or suppression of proliferation. Immunofluorescence staining of alpha smooth muscle actin fibers was used to indicate phenotype changes in the vascular smooth muscle cells.

VSMCs were treated with varying concentrations of both heparin loaded and unloaded amine terminated particles varying from 0-100 µg Fe/ml. MTS assay done on amine terminated particles showed some amount of cell death at all concentrations (figure 3 B). Heparin coated particles showed an increasing inhibition of VSMC cell proliferation starting at 1 µg Fe/ml (p-value = 0.0007) with an increasing inhibitory effect up to 100 µg Fe/ml (p-value = 0.0006) where effects measured by the MTS assay were indistinguishable from the amine coated particles (figure 3 A).
Figure 3. MTS assay of 3 cell lines commonly associated with neointimal hyperplasia incubated with A) heparin coated nanoparticles, B) primary amine terminal nanoparticles, C) pure heparin.
It is important to note that MTS proliferation assays were done using particles with a heparin loading of 0.976 µg of heparin/µg Fe. Live/dead assays (figure 3) were also performed as a secondary indication of cell viability. Amine terminated particles showed a significant decrease in cell viability at particle concentrations of 25 µg Fe/ml (figure 3B) where the heparin coated particles showed a significant reduction in cell viability at 10 µg Fe/ml (figure 3A). Although initially MTS and live/dead assay results for the heparin loaded particles are not seemingly in agreement it is again important to note that the live/dead assays were done using a different batch of synthesized particles with a much higher loading of heparin (2.89 µg heparin/µg Fe). Both assays indicate however that heparin concentrations above 10 µg lead to significantly lowered cell viability. Live/dead assays done on the heparin coated particles showed that the reduction seen in the MTS assay was due to suppression in proliferation and not cell death. Comparatively both MTS and Live dead assays on VSMCs incubated with up to 3200 µg/ml of heparin showed no effects on the resulting cell viability as seen in figure 3 C and figure 4 C. Although reports of as little as 25 µg heparin/ml inhibiting the proliferation of VSMCs exist, it is important to note that all low molecular weight heparin does not show the same chemical, physical and biological properties. This may be due to the different starting source materials and chemical processing (depolymerization). The pure heparin experiments in this manuscript were done using the same heparin used in the particle conjugation as to avoid erroneous results due to the variance in low molecular weight heparin from different manufacturers.
Figure 4. Live/dead assay of 3 cell lines commonly associated with neointimal hyperplasia incubated with A) heparin coated nanoparticles, B) primary amine terminal nanoparticles, C) pure heparin.
Immunofluorescence imaging was used to observe VSMC morphology and visual trends in marker expression for each cell line. Figure 5 shows one sample image from each culture condition. Cell number was quantified over several images in the culture and compared to the MTS data and the trends were similar. Immunofluorescence staining of alpha smooth muscle actin (figure 5) was used to indicate the VSMCs lineage towards the synthetic or contractile phenotype. VSMCs in their synthetic phenotype typically exhibit a rhomboid shape with randomly oriented fibers. When in the contractile phenotype VSMCs have a spindle shape with contractile apparatuses aligned into organized fibers. Incubation with amine functional particles in concentration of both 1 and 10 µg Fe/ml showed disruption of normal cell behavior resulting in cell fragmentation. It is interesting that the nuclei have seemingly been removed/destroyed, as it is not seen in the stained samples. VSMCs incubated with heparin-coated particles at both concentrations of 1 and 25 µg Fe/ml exhibited preference towards the contractile phenotype as shown by the organized actin distribution and spindle shape associated with the quiescent state. Immunofluorescence staining of the PECs and fibroblasts showed no change in expression of VE-cadherin or collagen I.
Figure 5. Immunofluorescence staining of relevant cell lines to observe phenotypic changes. Top row shows VSMCs. Smooth muscle actin is seen in green and nuclei are seen in blue. In the middle row endothelial cells are shown with VE-cadherin in cellular junctions. 3T3 fibroblasts were stained for collagen I.

Endothelial cells play a vital role in the formation of the tunica intima and it is important to observe effects due to interaction with the particles as proper endothelialization is needed for proper healing. MTS and live/dead assays were run on
porcine endothelial cells incubated with the same varying concentration of both the amine terminated particles and the heparin coated particles. MTS on endothelial cells incubated with the amine terminated particles (figure 3 B) showed a pattern of increased proliferation leading to an increase in viability with a sudden drop to zero at 50 µg Fe/ml (p-value < 0.0001). The MTS after incubation with heparin coated particles showed the same trend of increased cell viability due to an increase in proliferation up through the 100 µg Fe/ml (p-value = 0.0083), which was the highest concentration of particles used in this study (figure 3 A). The same increase in viability was also seen with raw heparin at concentrations between 1.56 and 1600 µg/ml (figure 3 C). Live/dead assays conducted using amine terminated particles showed relatively stable cell viability between 90% and 120% until a major drop to 0% is seen at 50 µg Fe/ml (figure 4 B). This is consistent with the MTS assay and shows that at concentrations above 50 µg Fe/ml the amine terminated particles induce cell death and nonproliferation. Live/dead assays of the endothelial cells incubated with the heparin coated particles showed a lowering of the cell viability from 100% to approximately 60% at 10 µg Fe/ml, with higher concentrations of particles (up to 100 µg Fe/ml) maintaining the 60% cell viability seen at 10 µg Fe/ml (figure 4 A). Live/dead assays after incubation with pure heparin showed no discernable trend in cell viability up to 1600 µg/ml (figure 4 C).

MTS and live/dead assays were also performed on 3T3 mouse fibroblasts. Fibroblasts take a passive role in the healing of intimal hyperplasia so relative lack of response to the administered treatment is preferential. MTS of fibroblast cells treated with amine terminated particles showed relatively stable cell viability between 80-100% up to
25 µg Fe/ml (p-value = 0.0002) where cell viability sharply drops to 15% and further to 0% at 50 µg Fe/ml (p-value = 0.0002) and 100 µg Fe/ml (p-value = 0.0002) (figure 3 B). Fibroblasts treated with heparin coated particles show a relatively stable cell viability throughout incubation with all particle concentrations with no discernable trend in viability (figure 3 A). The cell viability of fibroblasts incubated with pure heparin up to 1600 µg/ml show slightly erratic behavior with no real trend (figure 3 C). Live/dead assays done on fibroblasts incubated with amine terminated particles confirms the behavior seen in the MTS assay although the viability seen from live/dead immediately drops to approximately 50% at incubation with only 1 µg Fe/ml and drops further to 0% with 25 µg Fe/ml up to 100 µg Fe/ml (figure 4 B). Live/dead assays done using heparin coated particles agreed with the MTS assay done as no discernable trend was seen. A slight decrease in cell viability to 50% was seen at both 10 µg Fe/ml and 25 µg Fe/ml but at concentrations above 25 µg Fe/ml cell viability was back above 80% (figure 4 A). Live/dead assays done on fibroblasts incubated with only heparin showed a slight decreasing trend from 100% viability to approximately 65 percent viability at the highest concentration of heparin used (1600 µg/ml) (figure 4C).

Magnetite nanoparticle uptake by the cells was observed using TEM (figure 6). Excess particles were washed before fixation to get rid of any particles that were not up taken by the cells. After embedding the cells, they were sectioned into 70-90 nm thick sections. The images show a cross-section inside the cells. As observed in the images, particles were found mainly in the cytoplasm of the cells either individually or clustered in
endosomes. The images also suggest that the particles are likely internalized by the cell through pinocytosis (figure 5 B arrow).

Figure 6. A) TEM image (scale bar = 2 µm) of rat vascular fibroblasts treated with magnetite nanoparticles. Nanoparticles internalized in endosomes. B) TEM image (scale bar = 500nm). C) EDX of cells showing appearance if iron peaks at 6.4 and 7.1 KeV (circled in red).

The presence of iron was assessed using Energy Dispersive X-Ray Analysis (EDAX) microanalysis system; data shown in figure 6C. A large Fe peak was seen in the
cellular sample indicating uptake of the particles into the cell and not simple attachment to the outer cellular membrane.

Three female athymic nude mice were in injected IV with 100 µl of approximately 250 µg Fe/ml in buffer. Mice were MR imaged at 9.4T pre-injection, 20 min post injection and 1.5 hours later. T1- and T2- weighted images were collected. No physiological reactions to the injection were observed acutely in the three imaged animals. No adverse reactions were observed post injection in the animal that was sacrificed one week later. No significant changes were observed in the T1-weighted images. T2-weighted images showed significant hyperintensity (signal decrease) 20 min post injection in highly vascularized regions including the kidneys, brain, and heart (Table 1). This was expected with the particles as magnetite is used as a T2 contrast agent. The hyperintensity signal intensity reported in table 1 was calculated from the specific regions of interest shown in figure 7.

Table 1. Mean intensity of ROIs in arbitrary units pre- and 20 min post injection. ROIs were circular with a radius of 1.8 mm. ROIs 1 and 3 were in the kidneys and ROI 2 was in the brain.

<table>
<thead>
<tr>
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<td>3</td>
<td>8177</td>
<td>603</td>
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Figure 7. T2-weighted MR images of mouse injected IV with 100 µl of 200 µg Fe/ml in buffer. (a) pre-injection. (b) 20 min post injection.

Image intensities returned to baseline at 1.5 hours. Although not a quantitative indication of high circulation times, MRI results suggest that the heparin loaded particles are in circulation and cleared after 1.5 hrs. This shows that unlike conventional heparin which binds heavily to cell surfaces, the particles maintain circulation instead of being immobilized at the injection site. This is promising as it may allow intravenous administration of the heparin coated particles without high amounts of rapid unwanted binding to cellular sites.
Although encouraging, further studies are needed to construct a more accurate and validated pharmacokinetic profile of the heparin coated nanoparticles. This should include fluorescent tagging of the particles and ICP-OES of major organs, blood and excrement to look at iron content and circulating nanoparticle concentration at varying time points after administration. This can be correlated with the hyperintensity MR signal to construct a more complete understanding of the \textit{in vivo} behavior. $^{59}$\textit{Fe} Radio labeled Fe$_3$O$_4$ or $^{13}$\textit{C} labeled PEG could also be used to validate and further confirm the biodistribution, retention, and excretion of the nanomaterial.\textsuperscript{49}

Conclusions

Heparin, both administered locally and systemically, has been used for years as an anticoagulant. Studies have shown that heparin has antiproliferation properties but it has not been used clinically due to the large dosage needed as heparin is cleared quickly \textit{in-vivo}. Results in this manuscript show that magnetic nanoparticles coated in heparin can achieve the antiproliferative activity of bulk heparin at much lower doses, in part due to the large surface area to volume ratio of the particles leading to high heparin/particle loading. Restriction on the proliferation of vascular smooth muscle cells due to phenotypic changes was seen with concentrations of heparin coated particles as low as 1 $\mu$g of Fe/ml. Heparin coated particles were shown to have a proliferative effect on endothelial cells. While pure heparin also induces proliferation in endothelial cells, comparative heparin concentrations to achieve this were much lower with the heparin coated particles. Fibroblast cells showed a relative lack of response to the heparin coated particles.
Anticoagulative properties of the heparin loaded nanoparticles will be investigated in future work to further characterize the potential effect on thrombi formation. Biodistribution via MRI showed relatively long circulation times *in-vivo* which is desirable for treatment of neointimal hyperplasia. Long circulation times are also paramount for possible use in magnetically targeted therapy using magnetically stents, as multiple passes through the stented area would increase the probability of magnetically attracting a heparin loaded particle to the stent, localizing the treatment to the stent area.
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CHAPTER FIVE

COMBINATION T1 AND T2 CONTRAST AGENTS FOR DUAL MODE MAGNETIC RESONANCE IMAGING.

Introduction

One of the largest fields where magnetic materials see usage is that of MRI contrast agents. Typically small molecule paramagnetic chelates will be employed as T1 shortening agents.\(^1\) Because these molecules diffuse rapidly and change the positive contrast signal they are widely employed in the medical field to detect lesions etc. where multiple environments may have a similar Larmor frequency and therefore are indistinguishable from each other in a non-contrast enhanced scan.\(^2\) These mainly consist of gadolinium complexed with either linear ethylenediaminetetraacetic acid (EDTA) derivatives, or macrocyclic molecules such as 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid (DOTA), 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7-triacetic acid (DO3A), and their functional derivatives.\(^1\) Although toxic, gadolinium is a prime candidate for T1 contrast enhancement as it has a high magnetic moment due to its seven unpaired f electrons and a relatively long electronic relaxation time.\(^3\) The toxicity can also be significantly reduced by using the macrocyclic class of chelates vs the linear, as they have lower muscular deposition and are generally regarded as safer.\(^4\)

Magnetic nanoparticles, typically magnetite, are also used as contrast agents for MRI. However as was alluded to in the introductory chapter, the relaxation mechanism that they primarily affect is much different. Because the magnetic particles produce large inhomogeneities in the local field, they primarily effect the local field felt by the surrounding protons which massively changes the velocity of dephasing.\(^5\) Because of this
magnetic nanoparticles are thought of as T2 contrast agents. Although the magnitude of the change in T2 seen from particles is typically an order of magnitude greater than the change imparted on T1 by gadolinium, they are not favored by clinicians as they impart a negative contrast change, there are difficulties in biological half-life, and questionable side effects have been seen in some patients.\(^6\)

Each individual agent, either the Gd based chelates or magnetic nanoparticles, is useful when only a single mode of imaging is used. However, there is an inherent issue when using only T1 or T2 weighted imaging procedures. Each of the specific modalities can be heavily influenced by artifacts that show up in final scan images. These artifacts typically manifest from \textit{in vivo} phenomena such as calcification, clotting, fats, air, etc. and they may mimic the behavior of single mode contrast agents, or cause severe misrepresentation of the issue.\(^7\) These can be very problematic as they may lead to poor diagnostic accuracy, extended imaging procedures, and costly secondary scans including more scans with contrast. Shin et al. propose an interesting concept to eliminate the possibility of misinterpreting MR artifacts as administered contrast.\(^8\) They proposed that it is possible to use an AND gate after taking both T1 and T2 weighted images to eliminate artifacts seen on either of the single weighted image.\(^8\)
To do this however the contrast agent must fulfill multiple criteria that today’s clinically available agents do not satisfy. This includes a high relaxivity for both modes, as well as a relaxivity ratio that is small so T2 shortening does not detrimentally effect the T1 imaging process. By combining the shortening and relaxation effects of both magnetite nanoparticles and gadolinium chelates it may be possible to increase overall R1 relaxivity while conversely lowering the extremely high R2 from the magnetite.
The proposed dual mode contrast agent is represented in figure 1. This is one of the strategy’s outlined by Zhou et al. to create a dual mode contrast agent via inner and outer water shell management. This research looks at the relaxivity effects on water different size magnetite particles functionalized with gadolinium chelates using varying molecular weight poly(ethylene glycol) polymers as spacers. By systematically looking at different size cores and spacer lengths it is possible to elucidate the effects of the gadolinium functionalization and their effect on the R2/R1 ratio.
Experimental

Synthesis of PEO-PAA-Dopamine

Figure 2. Synthesis of multianchored alkyne functional polymer. 1) ring opening of ethylene oxide to for protected amine terminated poly(ethylene glycol) (PEG). 2) Alkylation of alcohol for alkyne functionality. 3) Deprotection of amine. 4+5) EDC chemistry to attach PEG and dopamine to poly(acrylic acid) backbone.

PEO synthesis: Ethylene oxide was distilled into a high pressure Parr reactor. Dry THF was injected along with a predetermined amount of an anionic initiator (in this case potassium bistrimethyl silyl amide). The reaction was allowed to run for 72 hours and
was subsequently terminated by opening the reactor to atmosphere. The synthesized PEO was precipitated with diethyl ether and washed (3x) by dispersing it in chloroform, precipitating the polymer, centrifuging it and pouring off the residual supernatant. The PEO was then dried under vacuum overnight. HNMR was performed to calculate molecular weight as well as to confirm the presence of the protected amine endgroup.

Under dry N$_2$ atmosphere heterofunctional PEO and sodium hydride (in slight excess) were dissolved in dry THF. This was allowed to react for 30 minutes before an excess of propargyl bromide was added dropwise to the solution over 15 minutes. Once all of the propargyl bromide was added, the solution was allowed to stir for 12 hours at room temperature. The polymer was then purified by dissolution in chloroform and precipitation with diethyl ether (x3) and dried under vacuum for 12 hours. HNMR was performed to confirm the presence of an alkyne.

Deprotection of the trimethyl silyl group was done in 1M Hydrochloric acid in methanol and allowed to react for 4 hours. The polymer methanol solution was diluted with water and the deprotected PEO was extracted (3x) with chloroform from which it was precipitated with diethyl ether and dried under vacuum. HNMR was performed to confirm the loss of the tri methyl silyl group.

Coupling of the PEO to the poly(acrylic acid) was done by dissolving both in dry DMF in a 5:1 ratio. To this 1.1 excess EDC as well as catalytic amounts of DMAP were added. The solution was allowed to stir for 12 hours. The solution was filtered, further purified by dissolution in chloroform following precipitation with diethyl ether (x3) and then dried under vacuum. HNMR was done to confirm PAA-PEO coupling.
Attachment of the anchor group: Dopamine hydrochloride was dissolved in THY along with 1.1 excess triethylamine and allowed to stir for 30 minutes. In a separate round-bottom the PEO-PAA was dissolved in THF along with EDC and catalytic amounts of DMAP. To this the dopamine hydrochloride solution was added and the combined solution was allowed to stir for 12 hours. The solution was then filtered, purified by dissolution in chloroform then precipitated in diethyl ether. The final product was dried under vacuum and analyzed via HNMR (Figure 3) and IR to confirm the presence of the catechol.
Figure 3. Representative HNMR of final polymer after full modification m-multiplet, q-quartet, t-triplet, b-broad. 0 ppm = tms, (m) 0.75-1.5 ppm = PAA, (q) 2.42 ppm and (t) 4.18 ppm = alkyne, (b) 2.8 ppm = alcohol, (b,m) 3.62 ppm = PEG.

Synthesis of magnetite nanoparticles

Magnetite nanoparticles were synthesized via thermal decomposition of an organometallic precursor in a high boiling point organic solvent. Iron (III) acetylacetonate (1.074 g) was combined in a 3 neck round bottom with varying amounts of oleic acid and octadecene depending on the desired particle size. The vessel was
initially purged with N₂ after which flow was adjusted to 0.1 L/min ensuring an inert environment. The vessel was then heated to 350 °C and left to react for 3 hours. At 3 hours the reaction was quenched by removing from heat, and left to cool under inert atmosphere. The resulting particles were dispersed in minimal hexanes and precipitated using a mixture of 3:1 ethanol to acetone (x3). Particles were dispersed in toluene and run through an organic based GPC column to further remove excess ligand. TEM and size analysis was then done on the particles to ensure size specificity.

Figure 4. Particles of four different sizes used for the gadolinium functionalization ranging from 14.9nm to 34.3nm.

Synthesis of clickable chelate

Azido-DOTA complex was synthesized in accordance with Szijjártó et al. ²⁻ azidoethanol and triethylamine were dissolved in dichloromethane (DCM) under an inert N₂ atmosphere. The solution was cooled in an ice bath and methanesulfonyl chloride was added dropwise via syringe. The reaction was stirred for two hours. After two hours dilute HCL aq. was added to solution. The phases were separated and the aqueous phase was extracted once with fresh DCM. The organic phases were combined and dried with
Evaporation of the solvent afforded an oily yellow liquid final product of mesylated azidoethanol.

Mesylated azidoethanol was added dropwise to a suspension of excess cyclen in chloroform. The solution was stirred for 24 hours at room temperature and allowed to react. The reaction was then concentrated and purified via a gradient flash chromatography (0 %-15 % DCM in methanol then 10:10:1-5:5:1 DCM-MeOH-saturated aq. NH$_3$. Final monoalkylated cyclen product was a pale yellow oil and was verified via HNMR.

Monoalkylated cyclen and Na$_2$CO$_3$ were suspended in acetonitrile (ACN). Tert-butyl bromoacetate was added to the reaction and the mixture was heated to 70°C for 24 hours. The mixture was filtered, concentrated and purified via flash column. The final product was a pale yellow oil which was verified by HNMR.

Deprotection of the tert-butyl groups was done in a 1:1 solution of triflouro acetic acid and dichloromethane by stirring at room temperature for 24 hours. The volatile byproduct and solvent was evaporated off and the resulting product was dried for 48 hours in a vacuum oven at ambient temperature. The final product was a sticky yellow liquid and was verified via HNMR and IR for the correct functional groups.

Chelation of the gadolinium was performed in methanol my mixing a 1:1.05 equivalent of the chelate to GdCl$_3$. The mixture was heated to 45 °C for 24 hours. The mixture was then poured into diethyl ether and the precipitate was collected. The precipitate was once more dissolved in MeOH and precipitated for purification. The solid precipitate was dried under vacuum for 48 hours prior to analysis via FTIR (figure 5).
Figure 5. FTIR of azide functional DO3A based gadolinium chelate used to functionalize magnetic particles.

Ligand exchange

Both magnetite nanoparticles as well as the PEO-PAA-dopamine were suspended separately in 5ml of chloroform. The particles at an approximate concentration of 3 mg/ml of Fe and the polymer at approximately 40 mg/ml. The polymer was then transferred to a scintillation vial capped with a septum and placed in a sonication bath. The bath was turned on and over the course of 15 minutes the magnetic nanoparticle solution was injected into the polymer solution. Once injection was finished, the combined solution was allowed to further sonicate for 15 minutes. The solution was then removed and put on a shaker table for 72 hours. The chloroform was then removed via rotary evaporator and further dried under vacuum. DI H$_2$O was then added and the vile
was sonicated to help mediate dissolution into the water. The water based particles were then filtered through a 0.2 micron nylon filter to ensure large aggregates were not present. The solutions were then run through a GPC column to separate excess polymer from the water suspendable particles. Further purification was done using both centrifugation and rare earth magnets to pull particles out of solution, decant the supernatant, and redisperse in DI H₂O.

THPTA synthesis adapted from Hong et al.¹³

Synthesis of bis-triphenylphosphine complex of Cu(I)acetate:

Cu(I)acetate (1 g) and triphenylphosphine (4.3 g) were combined in a round bottom flask and purged with dry N₂. 50 ml of dry dichloromethane was added to dissolve the reagents and the reaction was allowed to stir for 1 hour. The contents were then filtered over cellite and the resulting colorless solution was immediately poured into 500ml of ethyl ether and put in the freezer for 12 hours to crystalize. The product was then filtered and dried under vacuum.

Synthesis of 3-azido-1-propanol:

3-bromo-1-propanol (20 g) and sodium azide (18.7 g) were dissolved in DI H₂O and heated to 50°C for 24 hours. The product was then extracted (3x) with dichloromethane and dried over anhydrous magnesium sulfate. After filtration the mixture was concentrated and purified via fractional vacuum distillation yielding a viscous clear colorless liquid. HNMR was done to verify the final product.
THPTA synthesis:
Tripropargyl amine (1g) and 3-azido-1-propanol (3.075g) were combined in 50ml of
dried dichloromethane and the reaction vessel was purged with dry N₂.
Bistriphenylphosphine Cu(I) acetate (98.5mg) was then added using positive N₂ pressure.
The reaction was cooled in an ice bath for 4 hours. The reaction was then warmed to
room temperature and stirred for 24 hours. After the initial 28 hours the reaction was
charged with a secondary amount of both 3-azido-1-propanol (1.2g) and
bistriphenylphosphine Cu(I) acetate (98.5mg) and left to stir for another 24 hours.
Dichloromethane (50ml), H₂O (50ml) and saturated EDTA solution (50ml) was added to
the reaction and then transferred to a seperatory funnel. The reaction was washed with
dichloromethane (4x) and the aqueous layer was collected and lyophilized. The product
was then dissolved in a minimal amount of 80:20 dichloromethane to methanol and
passed over activated alumina using 200 ml of the 80:20 dichloromethane to methanol as
an eluent. The colorless and clear eluent was collected, reduced in volume, poured into
300ml of diethyl ether and left to recrystallize in the freezer for 24 hours. The resulting
product was filtered and dried under vacuum. HNMR was performed to verify the
product.

Click Chemistry
PEO modified particles were suspended in DI water along with excess functional
gadolinium chelate. Copper (II) sulfate was dissolved separately in DI water with
equivalent molar amounts of THPTA ligand. The THPTA-copper sulfate solution was
added into the particle-Gd chelate solution until the copper concentration was 2 mol percent of the chelate. A fresh solution of sodium ascorbate was then added up to 10 mol percent to reduce the copper to copper (I). The reactions were left to react for 8 hours. Excess reactants and copper were removed via gel permeation chromatography and dialysis in DI water.

Relaxivity Measurements

Relaxivity measurements were taken at 4 different field frequencies at 37°C using four different Bruker Minispec relaxometers fitted with a circulating water bath for temperature control. T1 relaxation measurements were done using a typical inversion recovery sequence, 10 data points were used to fit T1 relaxation with the largest duration being up to 1000ms. T2 was measured using a multiecho spin echo sequence. 10 echoes ranging from 5 ms to 500 ms were used to fit the T2 relaxation. Both T1 and T2 fitting was done using minispec Plus software, reporting the T1 and T2 values.

Inductively Coupled Plasma Optical Emission Spectroscopy

Samples were digested initially with boiling peroxide to try and eliminate organic from the surface. Once boiled off, concentrated nitric acid was added and boiled to dissolve all metals from the particle complexes. Once dissolved the samples were diluted with 2% nitric acid at a known volume. Samples were given to ChemCentre at the Curtin University of Technology to analyze via ICP-OES.
Results and Discussion

Chaining Effects in Magnetic Field and Magnetite Relaxivity

Initial evaluation of the contrast agents was not for their relaxivity values, but more to confirm their behavior in a large magnetic field. Chaining of magnetic nanoparticles inside of a field has been observed by Saville et al. who showed that the relaxivity decreases as a function of time as the particles assemble into linear aggregates inside of the field. To examine if the PEG Fe₃O₄ system used for these experiments exhibited the same behavior, the T2 values of a concentrated aqueous solution of 30nm particles coated with a 17k Mw PEG was measured over 10 minutes inside a 60MHz field. As can be seen in figure 6, no change in the T2 value was measured over the course of 10 minutes.
Figure 6. T2 vs time curve of aqueous solution of 30nm Fe₃O₄ coated with 17k Mw PEO at a concentration of ~1.5mg Fe/ml. T2 measurements were run at a field strength of 60MHz.

It can only be said however that we are in some state of equilibrium as the sample temperature had to equilibrate within the field for up to 2 minutes. During this time any sort of chain assembly may have happened. It is important however that we did not see any change in the T2 signal after temperature equilibration, as the samples relaxivity values after equilibration do not depend on time. This allows for comparison of the relaxivity values without influence from large mesoscale assembly.

Although initial experiments into the stability of the particles showed that chain formation may be minimal, measured R2 values for the magnetite particles in water showed that chain formation/mesoscale assembly was probable within the equilibration period. Figure 7 shows the R2 values for different core size magnetite particles modified with varying molecular weight PEO brushes at different field strengths.
Figure 7. R2 values of PEO stabilized magnetite nanoparticles at multiple field strengths. Relaxivity goes down as particle size increases and/or stabilizer molecular weight decreases. Red = 15nm, Green = 20nm, Black = 25nm, Yellow = 30nm.

As both the molecular weight of the spacer and the core size go up, the relaxivity values decrease. Initially this is counterintuitive as the larger particles should have a much larger magnetic inhomogeneity effect, however again the results are supportive of those reported by Saville et al. the organization of particles into larger structures negatively impacts the R2 values. This is in line with the observations seen here as smaller particles with larger brushes are the least prone to in field organization as the moments are much smaller and the steric stabilization may be able to overcome the self-
assembly forces. Any chaining or mesoscale organization process may happen at relatively short time scales, and although unavoidable, it is important to let come to some equilibrium state before evaluating particle based contrast agents as quick measurements may skew the relaxivity values.

Unlike the R2 which is affected by the core size and PEO spacer due to chaining phenomena, the R1 of the magnetite samples does not seem to follow any such trend. As was expected, the R1 of the magnetite samples is drastically lowered at higher fields. This is one of the reasons that particles (especially of this size), cannot be used at T1 contrast agents in general. Typical MRIs operate at between 40 and 130 MHz, at these operational fields the R1 is drastically lowered. Combining this with a relatively stable or increasing R2 as the field increases, the R2/R1 ratio increases dramatically making any sort of dual mode imaging with purely magnetite particles impossible. This change in the R1 vs. field can be seen in figure 8.
Figure 8. R1 values of PEO stabilized magnetite nanoparticles at multiple field strengths.

*R1 relaxivity drastically decreases as the field increases. This is problematic for dual mode imaging as it increases the R2/R1 ratio.*

Although there is an obvious field dependence of R1, there is no trend relating to the particle size or PEO spacer on the R1 behavior of the pure magnetite particles.

**Coupled Gadolinium and Magnetite**

A lot of research has gone into the synthesis of functional gadolinium chelates so they can be further modified with other moieties including polymers, small molecules, etc. There are specifically two different routes of synthesis that have been used to make chelate molecules for this research as is illustrated in figure 9.
Figure 9. Two separate routes to prepare monofunctional gadolinium DO3A chelate.

Rout 1 is significantly more difficult as there are large gradient flash columns that need to run on the compound to separate out any of the di, tri, or tetra alkylated compounds. Synthesis rout 1 does have its advantages relating to the ability to get the multi alkylated complexes if it was wanted to use as a step growth monomer or to build larger macroscale complexes off of the chelators. Reaction 2 shown in figure 2 is much easier to purify so larger quantities can be obtained quickly. However multi-functional chelates cannot be prepared this way as it is the trialkylated complexes that salt out of solution, which still leaves a separations issue.

To affect the R1 properties of the particles, gadolinium chelates, prepared via rout 1, were “clicked” onto the end of the stabilizing ligand. This allows for the surrounding
water protons to easily interact with the gadolinium which will generate a measurable enhancement in R1. Table 1 shows the change in the relaxivity values for the particle after coupling with the gadolinium chelate. The table is only showing the 60 MHz field strength however the trends seen are similar at all field strengths. The percent relaxivity change is highlighted in yellow.

Table 1. Relaxivity values at 60MHz before and after Gd is added to the system.

<table>
<thead>
<tr>
<th>*60 MHz Units in (mM⁻¹s⁻¹)</th>
<th>No Gd R1</th>
<th>With Gd R1</th>
<th>% change</th>
<th>No Gd R2</th>
<th>With Gd R2</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>17k 15nm</td>
<td>3.73</td>
<td>9.48</td>
<td>154.1555</td>
<td>282.34</td>
<td>217.3</td>
<td>-23.0361</td>
</tr>
<tr>
<td>17k 20nm</td>
<td>3.834</td>
<td>--</td>
<td>--</td>
<td>191.85</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>17k 25nm</td>
<td>5.739</td>
<td>6.87</td>
<td>19.70727</td>
<td>193.97</td>
<td>197.9</td>
<td>2.026087</td>
</tr>
<tr>
<td>17 30nm</td>
<td>3.382</td>
<td>5.19</td>
<td>53.45949</td>
<td>215.52</td>
<td>211.78</td>
<td>-1.73534</td>
</tr>
<tr>
<td>7k 15nm</td>
<td>2.6303</td>
<td>4.48</td>
<td>70.32278</td>
<td>209.43</td>
<td>157.3</td>
<td>-24.8914</td>
</tr>
<tr>
<td>7k 20nm</td>
<td>2.4888</td>
<td>3.86</td>
<td>55.09482</td>
<td>183.73</td>
<td>153.8</td>
<td>-16.2902</td>
</tr>
<tr>
<td>7k 25nm</td>
<td>2.277</td>
<td>--</td>
<td>--</td>
<td>181.47</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7k 30nm</td>
<td>1.586</td>
<td>--</td>
<td>--</td>
<td>127.03</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5.5k 15nm</td>
<td>2.81</td>
<td>3.89</td>
<td>38.43416</td>
<td>214.3</td>
<td>155</td>
<td>-27.6715</td>
</tr>
<tr>
<td>5.5k 25nm</td>
<td>3.143</td>
<td>2.43</td>
<td>-22.6853</td>
<td>156.96</td>
<td>88.8</td>
<td>-43.4251</td>
</tr>
</tbody>
</table>
It is interesting to note that with most of the samples, there was a positive change in the R1 relaxivity which was expected due to the presence of gadolinium in the system. What was not expected was the negative impact on the R2 signal. This negative change actually works in favor for creating a dual mode agent as the R2 signal of magnetic nanoparticles are high enough that lowering the R2 is a viable option to change the relaxivity ration. In this case the gadolinium coupling works two fold by lowering the R2 and increasing the R1. The normalization process for complex systems such as these can be difficult.

In this case it is important to note that the relaxivity values reported for the coupled magnetite-gadolinium was obtained by normalizing to all of the magnetic content. These values would be much higher if per mM of particles was used versus total amount of magnetic material. The decrease in the R2 signal may be due to the fact that the gadolinium contribution compared to the magnetite is very small, and therefore by increasing the normalization concentration we are lowering the R2. It is also important to note that the amount of gadolinium bound to the surface of each particle was not highly controlled so trends are most important not necessarily the individual numbers as they may vary depending on the gadolinium loading.

The R1 of the gadolinium loaded magnetite particles shows an interesting trend in that the relaxivity magnitude is dependent on both the core size and the PEO spacer. This shows that the contribution to the relaxivity of the gadolinium is not simple as the local field from the magnetic particle influences the behavior of the gadolinium. This trend is
illustrated in figure 10 where we see the smallest particle of 15nm with the largest PEO spacer of 17k Mw has the largest R1 relaxivity.

As the particle core size gets larger the R1 relaxivity drops. This is akin to increasing the local field influence on the gadolinium chelate. The same behavior is seen as the PEO spacer decreases in molecular weight, which again is akin relates to the local field that interacts with the gadolinium. This phenomena was recently looked at by Choi et al. who use the influence of the magnetic particle on the gadolinium complex as a molecular ruler.\textsuperscript{15} Only a small discussion on the mechanism behind this phenomena is

\textit{Figure 10. R1 values after particles have been functionalized with Gd chelates. Trend shows that smaller particles with larger spacers tend to see the largest R1 gains.}
discussed, but the theory is that the electron spin fluctuation of the gadolinium chelate is
slowed via interaction with the magnetic particle. This slowing of the electron spin
fluctuation lowers the probability that electron-nuclear spin coupling occurs and therefore
reduces the R1 signal when the gadolinium is close to the magnetic particle.\textsuperscript{15} The use as
a magnetic alternative to FRET is definitely exciting, however this also can be used as a
guideline to increase the total R1 relaxivity of the complexes. By increasing the
molecular weight and therefore the distance of the paramagnetic chelate from the particle,
the R1 signal should heavily increase. What is still unknown is how larger distances fully
effect the coupling and where the optimal threshold is for increasing the R1.

Also promising is that the lowering of the R2/R1 ratio seems to be field
dependent, in that as the field increases we see larger changes towards a more reasonable
ratio. This is illustrated in figure 11 where the relaxivity ratio at larger fields is more
dramatically effected than at lower fields.
Figure 11. Calculated R2/R1 ratios of the particles pre Gd functionalization (circles), and post Gd functionalization (triangles). Magnitude of change between fields is altered when Gd is present.
That there seems to be a larger effect at larger fields is promising for possible use as a dual T1 and T2 contrast agent as most MRI scanners operate at or above 1T. The lowering of the ratio also seems dependent on the PEO spacer. Where the longer the spacer the larger the change in the ratio. This is explained by the larger change in T1 due to less interaction from the magnetite particle on the gadolinium. The ratios from the measured samples were still a bit large to be truly useful for dual mode imaging. However the trends seen illustrate that the use of a small particle to reduce the T2, and a large spacer coupled with gadolinium would further reduce the ratio into the regime that dual mode imaging would be possible, as well as maintain relatively high relaxivity values for both modalities.

Conclusions

Gadolinium functionalized magnetic nanoparticles of varying molecular weight and PEG spacer length were successfully synthesized. Complexes showed stable relaxivity reading in field which suggests that any mesoscale ordering has already occurred and is not influencing the measurements as a function of time. It was apparent that ordering was occurring as the smaller magnetite core size showed a larger relaxivity than the larger core samples. This is counterintuitive to theory, but is explained by mesoscale chaining which effects the relaxivity in a negative manor. As was expected T1 behavior was only influenced by field for pure magnetite. The T1 behavior of the gadolinium functionalized magnetite was much more complex. Trends follow that the
larger the core and the closer the gadolinium was to the core, the lesser the enhancement in T1 from the gadolinium. This implies magnetic interaction with the electronic spin of the gadolinium which influences the relaxation rate of the water. Because this phenomena is both core size and spacer length specific, it may be employed to possibly create functional contrast agents where the intermolecular spacing between the gadolinium pendent group and the magnetite core change as a function of environment and therefore a shift in the T1 will be see. Moreover as the particle size decreases and the PEG tether molecular weight increases, a lowering relaxivity ratio is seen both due to a lowering of apparent T2 and an increase in the T1. This is promising as a sufficiently small particle with a sufficiently spaced gadolinium chelate connected may reduce the ratio enough so dual mode T1-T2 imaging is possible.
References


CHAPTER SIX

OPTIMIZATION OF ROTATIONAL COORELATION TIMES OF T1 CONTRAST AGENTS VIA POLY(ETHYLENE GLYCOL) CONJUGTION

Introduction

Magnetic resonance imaging (MRI) is a critical aspect of modern medicinal practices, providing a noninvasive method to view the structure of the human body. In addition to being noninvasive, MRI does not use ionizing radiation and provides drastic contrast between different kinds of tissues, making it ideal in the fields of neurology, cardiology, and oncology, among others.\(^1\) To increase the utility of MR imaging, contrast agents (CAs) are used to further enhance the visual differences between tissues, as well as highlight blood flow and determine organ health. It is currently estimated that one in three MRI scans are contrast enhanced.\(^2\)

The vast majority of Food and Drug Administration (FDA) approved CAs in clinical use are gadolinium-based chelates. Gd contrast agents are administered intravenously, and are detected indirectly via their effects on the relaxation time constants (\(T_1\), \(T_2\), and \(T_2^*\)) of water in body tissues.\(^3\) By depressing the relaxation time, the CAs alter the signal intensity in the tissue regions they occupy, thus changing the contrast between affected and unaffected regions. This phenomenon is vital for many conditions as relaxation signals between healthy tissues and a malignancy may be similar resulting in minimal contrast difference in the final image. The agents also improve the visibility of inflammation, blood vessels, and tumors that would be otherwise difficult to see. Despite their widespread use, gadolinium-based CAs have been linked to the causation of nephrogenic systemic fibrosis (NSF).\(^4,5\) NSF is a disorder that is observed in patients
with renal failure and that manifests primarily in the hardening of the skin, but can affect virtually all body tissue and can cause severe handicaps and/or death.\textsuperscript{6, 7} The link between Gd-CAs and NSF was first reported in 2006, when five of nine patients in a study developed NSF within a month of undergoing an MR angiography that utilized Gd-DTPA, until then the most commonly used contrast agent containing gadolinium.\textsuperscript{8} To date, the majority of NSF cases have been linked to gadodiamide, which is one of the least stable gadolinium chelates available as a contrast agent.\textsuperscript{9} As a result, chelate stability has been linked to the cause of NSF, as unstable chelates are more likely to leach or become unstable, thus releasing toxic Gd\textsuperscript{3+} or causing sequestration.\textsuperscript{10} The toxicity concerns Gd chelates present have forced much development in the field to be focused on searching for low toxicity CAs.

Of particular interest in this respect are Gd chelates conjugated with macromolecules, which exhibit increased contrast efficiency, thus lowering the amount of chelate (and consequently, gadolinium) required for optimal results.\textsuperscript{1} The increased contrast efficiency that macromolecular CAs exhibit is due to their increased relaxivity that results from their larger molecular tumbling rate. The Solomon-Bloembergen-Morgan (SBM) theory of relaxation predicts that for every substance there is an ideal tumbling rate that produces the maximum relaxivity.\textsuperscript{11} Although not eliminating gadolinium entirely, if the contrast agents used in scans were specifically tailored for the scanner field strength, a lesser dose of the toxic agent would be required for the same enhancement in contrast. By conjugating different molecular weight poly(ethylene oxide) macromolecules onto the gadolinium chelates we can elucidate an optimal molecular
weight for a specific field. This is then compared to diffusion coefficients of the Poly(ethylene glycol) (PEG) itself in water to create a way to predict the optimal molecular weight for enhancement through NMR diffusion measurements. This would allow for all types of macromolecular contrast agents to be evaluated through diffusion measurements, and aid in the optimization of the rotational correlation frequency.

Experimental

End group modification of polymer

PEG polymers either monomethyl ether or diol terminated of varying molecular weights were obtained from Sigma Aldrich and were used without further purification. The polymers were dissolved in tetrahydrofuran and the alcohol end groups were deprotonated via sodium hydride and then reacted with propargyl bromide to yield alkyne functionality. The polymers were then precipitated with diethyl ether and dried in a vacuum oven to remove residual solvent. NMR was run to confirm the presence of an alkyne end group.

Synthesis of gadolinium chelate

Cyclen and a 3x excess of sodium acetate was suspended in N,N-dimethylacetamide at -20 °C. To this a 3.2X excess of tertbutyl bromoacetate was added drop wise over the course of 30 minutes. The reaction was allowed to come to room temperature and react for 24 hours. After the 24 hours the reaction was poured into water and stirred until clear. To the solution potassium bicarbonate was added until pDO3A
(protected DO3A) precipitated as a hydrogen bromide salt. The precipitate was filtered out, dissolved in chloroform washed with water, dried (via magnesium sulfate), and filtered once again. The chloroform volume was reduced and the purified pDO3A HBr salt was precipitated from diethyl ether. This was dried under vacuum to remove residual solvent.

The pDO3A was dissolved in water at 70°C then the temperature was reduced to 40°C. A 10% KOH solution was added and after 15 minutes the freebase DO3A was extracted 3x with hexanes which were combined and evaporated to yield a colorless viscous oil. This was precipitated in ether to yield a white powder. NMR was done to confirm the pDO3A compound.

pDO3A was dissolved in chloroform and was reacted with a 1.1 excess of Mesylated azido ethanol at 40 °C for 8 hours. Mesylated azidoethanol was prepared using the same procedure described in chapter 5. The alkylated product was concentrated and left under vacuum to yield a yellow viscous oil which was then suspended in a 1:1 mixture of dichloromethane and trifluoroacetic acid for deprotection for 8 hours, and then evaporated to an oil. HNMR showed the disappearance of the protecting tertbutyl groups. After deprotection the chelate molecule was stirred with equal amounts gadolinium chloride in methanol for 6 hours. The chelated gadolinium complex was precipitated in diethyl ether and then used with the alkyne terminal polymers for the click chemistry reactions.
T1 mapping of samples

All T1 and T2 mapping will be performed on a Siemens 3T Prisma clinical MRI using the Siemens built in T1 and T2 mapping protocols with the following parameters. T1-mapping: IR based with 250ms and 1200ms IR times. T2-mapping: multi echo, 32 echos from 30 to 960 ms. Both 3mm slices with in-plane resolution of 0.7 mm. Approximate protocol time 30 min including a high resolution T1 weighted MPRAGE scan (0.4 x 0.4 x 1 mm).

The Siemens protocols were validated for relaxation rate and relaxivity mapping using a MnCl$_2$ serial dilution calibration phantom. Relaxivity of the samples was determined in vitro using a 2:1 serial dilution of the agent with 8 dilutions.

ICP-OES of chelate-polymer solutions

ICP-OES was run on a Perkin Elmer Optima 3100 ICP-OES running WinLab32 version 3.1 analysis software. Known masses of gadolinium conjugated polymer samples were digested initially with <70% nitric acid. The nitric acid was boiled off and the resulting gadolinium nitrate salts were dissolved in a known mass of 2% nitric acid solution and run against standard solutions of gadolinium.

Diffusion NMR measurements

Diffusion measurements were done using a Bruker 300 MHz Nuclear Magnetic Resonance (NMR) instrument. Samples of different molecular weight polyethylene glycol in deuterium oxide were made in order to keep concentration consistent with
relaxation measurements. Approximately 0.5 mL of the sample was transferred into an NMR tube and inserted into the instrument. The sample was first run at 25 °C for 16 scans using the $^1$H NMR pulse program. The program was then changed to the diffusion measurement program, and the gradient length (p30) and diffusion time (d20) were optimized for each polymer. To do so, 16 scans were first run at p30=5000 μs and d20=0.1 s, both at a 2% and 95% gradient. The ideal peak height at g=95 is 10 ± 5% of the peak height at g=2. Once the diffusion parameters were optimized to achieve this ratio, additional NMR spectra were collected at gradients of 5%, 12%, 25%, 37%, 50%, 62%, and 87%. The diffusion coefficient was found from the slope of the decay curve after it was plotted using equation 1, which is analogous to the Stokes-Einstein equation.

\[
\text{Eq. 1 } I = I_0 e^{-D\gamma^2 g^2 \delta^2 (\Delta - \delta/3)}
\]

Where $I$ is the observed intensity, $I_0$ is the reference intensity, $D$ is the diffusion coefficient, $\gamma$ is the gyromagnetic ratio, $g$ is the gradient strength, $\delta$ is the length of the gradient, and $\Delta$ is the diffusion time.

Results and Discussion

Initially measurements on a simple chelate and a large multi-chelate macromolecule were made in conjunction with relaxivity measurements made on the gadolinium coated nanoparticles. The structures of the simple chelate and the large macromolecular structure can be seen in figure 1.
The most obvious difference between the two is the size/molecular weight. This plays a large role in the actual contrast mechanism as it influences the rotational correlation frequency. Another major difference due to the large covalently bound polymers will be the water binding kinetics. Due to possible steric hindrance water binding may be suboptimal. The last major difference between the two molecules is the number of gadolinium ions per molecule. In the case of the macromolecular complex there were approximately 5-7 gadoliniums per molecule versus only a single in the normal chelate. All of these factors have a large effect on the relaxivity of the contrast
agent in question. Figure 2 shows both the chelate (Figure 1A) and the large macromolecule (figure 1B), and their R1 and R2 relaxivities as a function of field strength.

![Graph showing R1 and R2 relaxivity for both the simple gadolinium chelate (red) and multi-gadolinium complex (blue) as a function of field strength. Structures are shown in fig.1.](image)

**Figure 2.** R1 and R2 relaxivity of both the simple gadolinium chelate (red) and multi-gadolinium complex (blue) as a function of field strength. Structures are shown in fig.1.

The major difference between the two agents is the magnitude of the relaxivities for both R1 and R2. The increase in R1 can be attributed to multiple gadolinium water binding sites per molecule, as well as the slowing of the molecular rotational frequency to better match that of the Larmor frequency of the proton. The R2 difference may be that the large amount of gadolinium occupying a small space creates large field inhomogeneity as a magnetic particle would. Another difference is the behavior as a
function of field. In R1 this is another manifestation of the molecular rotational correlation. Where there exists a Larmor frequency that matches with the rotational correlation and both above and below that frequency we expect a loss of signal. This phenomena is predicted by the Solomon-Bloembergen-Morgan (SBM) theory of relaxation which is illustrated in figure 3B.
Figure 3. A) Phenomena that effect the relaxivity of paramagnetic chelates which can be tuned to alter relaxivity values. B) Graphic illustration of SBM theory for how tumbling rate effects relaxation times. A lower T value is desired. Figure 3A was taken from Michael A Bruckman et al., Engineering Gd-loaded nanoparticles to enhance MRI sensitivity via T1 shortening, Nanotechnology, 24, 46, 2013, and reprinted with permissions from IOP Publishing. Figure 3B was reprinted courtesy of Allen D. Elster, MRIquestions.com.

Essentially SBM theory shows that there is a specific tumbling frequency where the relaxation time of water protons is maximized. This maximization yields the highest signal per gadolinium so it is of interest to optimize the tumbling rate. It is of note that since the optimal tumbling rate is correlated to the Larmor frequency, each field strength MRI should have a different optimal tumbling rate which can be controlled by the coupling of macromolecules of different sizes. Complexes are illustrated in figure 4.
Figure 4. Structure of gadolinium chelates coupled to varying molecular weight PEGs, which were used for relaxivity measurements.

To identify the optimal molecular weight for a 3T MRI a clickable gadolinium chelate was reacted with PEGs of varying molecular weights and their relaxivity measured. The relaxivity of these compounds is shown in figure 5. As the molecular weight increases the tumbling rate of the molecule is decreased and the relaxivity increases until tumbling slows beyond the Larmor frequency in which case relaxivity begins to decrease. The relaxivity of the molecular weight specific chelates is shown in figure 5.
After an initial drop in the relaxivity was seen with total molecular weights of approximately 1500 g/mol. This may be in part due to restriction of water molecule-gadolinium interaction which would slow the water exchange rate and lower the apparent relaxivity of the complex. However, after an initial drop in the relaxivity there is an increase until a peak relaxivity at approximately 5,000 g/mol comparable to the chelate itself and then another drop off in relaxivity. It is important to note that the drop off is not near the same rate of increase at low molecular weights as the x-axis in figure 2 is logarithmic. This is interesting as it is better to be on the larger side of optimal molecular weight than the smaller as the decrease is much less severe. It is also interesting to note that the optimal molecular weight in this case is close to the critical entanglement weight of PEG of approximately 4000 g/mol. This may play a role in the sudden downturn in the per gadolinium relaxivity as further steric interactions may be influencing the water binding, exchange, or rotational frequency all of which influence the overall relaxation.

Although the relaxivity values for the PEG conjugated chelates are, at their best, similar to that of clinically used gadolinium chelates, the trend seen can be used to optimize other more promising macromolecular chelates by looking at the diffusion coefficients of the macromolecules and comparing them to the relaxivity measured. Diffusion measurements of different molecular weight PEGs are shown in figure 6.
Figure 6. Diffusion coefficients of varying molecular weight PEGs measured via diffusion NMR. Change in diffusion coefficient slows as molecular weight increases.

Although diffusion is not a direct measurement of the rotational correlation frequency, the translational diffusion is similar to rotational diffusion as both derive from Einstein equations where particle radius and viscosity are important in determining the diffusion coefficients for both kinetic and rotational diffusion. As the molecular weight of the polymer increases the diffusivity (diffusion coefficient) decreases significantly initially but begins to level out at higher molecular weights. This is interesting as the rate
of change in the diffusivity as a function of molecular weight matches well with rate in change in the relaxivity. The diffusivity vs relaxivity graph which would amount to the diagnostic tool for macromolecular contrast agents is shown in figure 7.

![Figure 7. Generated “standard curve” showing the measured relaxivity as a function of measured diffusion coefficient.](image)

The slowed change in the diffusion coefficient at high molecular weights explains why over large changes in molecular weight after the peak relaxivity relatively small changes in the relaxivity are seen. It follows that if the change in the rotational correlation is relative to the change in translational diffusion, then at higher molecular weights the rotational component would also begin to slow in its variance with molecular weight leading to relaxivity closer to the optimal. Although not the sole reason for the enhanced
relaxivity values seen for macromolecular chelates in literature, this phenomena may play a part as it is beneficial to be on the larger side of optimal as the diffusion is slow to deviate from the optimal.

It may be possible to use this phenomenon to maximize the relaxivity of other macromolecular chelates. If the diffusion coefficient can be measured of the molecule in question it can be matched to the corresponding relaxivity value, which would be able to direct the synthesis procedure to make the complex larger or smaller. It would also be feasible to generate similar curves for other commonly used field strengths. This, in theory, would influence the optimal diffusion coefficient as if the field is lower the Larmor frequency would also be lower and therefore a slower rotation would be optimal and visa-versa with a larger field strength. When looking at the diffusion coefficient as a guideline for molecular weight it is very important to note that the rotational frequency is only one of many factors that influence the relaxivity and more research needs to be done to elucidate a set of rules and guidelines for synthesizing optimal paramagnetic macromolecular contrast agents.

Conclusion

This work establishes a simple method to evaluate and optimize the rotational frequency of novel contrast agents using diffusion behavior of PEG as a guide for optimal relaxivity enhancement. PEGs of varying molecular weights were successfully attached to gadolinium chelates via a robust click chemistry reaction. Initial hypothesis was that a marked improvement as tumbling slowed would be seen, however the macromolecules
had a negative effect on water shell kinetics and dynamics possibly due to steric hindrance or solvation. Although this was the case a clear trend of molecular weight vs. relaxivity was seen and was in agreement with the Solomon-Bloembergen-Morgan theory of relaxation with the only major difference being a much smaller change in relaxivity as the molecular weight increased above the optimal. Diffusion measurements of the PEG macromolecules were also done, and their behavior correlated with the observed changes in relaxivity. This diffusivity vs. relaxivity can be used as a tool to evaluate novel macromolecular contrast agents, and can be optimize for their rotational behavior to match a 3T magnet. This method could also be done for other magnetic field strength which ideally would lead to the development of field specific contrast agents, instead of the one size fits all approach we currently have in the medical imaging field today.

References


CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

The subject of magnetic nanomaterials, their synthesis, modification, and ultimately application still has an extremely large amount of undiscovered opportunity. Even today many of the “simple” subjects on the most basic of magnetic materials are changing in ways that will cascade through to the final applications based research that many scientists currently take part in. In this chapter I will discuss broad the broad conclusions of my research, future applications, as well as areas that may need focus, from a materials science perspective starting from the nanomaterials synthesis through the final application of these novel materials.

Synthesis

One of the most difficult problems in synthesis of nanomaterials to solve is not necessarily creating the material, but being able to create the material reproducibly. Many of these novel particles rely on properties that may deviate greatly if the particles in question are a few nanometers different in size or composition. This is especially true when talking about magnetic nanomaterials. Because two of the major defining traits of magnetic particles (relaxation time and magnetocrystalline anisotropy) are so heavily reliant on the size, shape, and composition of the particles. It is not just imperative that
there is a synthesis, but that the synthesis is reproducible in a way that the particles can be “manufactured” in high enough quantities so they can be further used for downstream engineering problems. As of right now, scale up of highly monodisperse, highly crystalline magnetic nanoparticles is difficult. Fluctuation in temperatures within large reaction vessels has proven to affect the morphology of the particles yielding faceted nanocrystals looking more like pebbles than spheres. These issues are pervasive in the nanoparticle community, and it is one of the reasons that the extended LaMer synthesis is so valuable. The reproducibility of the synthesis is very high for magnetite, and drips could be run continuously as to have mass production in a small scale reactor, but it is important to evaluate the synthesis procedure when transitioning to other more complex materials such as nonstoichiometric ferrites.

As was seen in chapter 2 the transition to a mixed ferrite material such as manganese ferrite, nickel ferrite, or zinc ferrite using a simple one pot synthesis was not a simple follow the magnetite procedure. Different sizes, and morphologies were obtained using the same synthetic procedure. This is not to say that this will not work with minor alterations, or with a chosen material just that it is important to thoroughly investigate the effect of a certain synthesis procedure on the resulting material.

Another promising line of research that may be undervalued in the nanosynthesis community is the use of simulation to help narrow materials space. If the idea is to synthesize materials that are useful for engineering purposes, optimization of the ideal parameters is crucial. Using conventional wet methods it is difficult to predict or even identify the effect of doping on the materials properties. This was seen with the cobalt
ferrite where the AC magnetometry peak loss frequency was maximized at a nonstoichiometric value. Without completely characterizing a large set of reactions this behavior would not be seen. This is even more complicated when multiple dopant elements are introduced into the system as the materials space grows exponentially. Using simulation, it may be possible to narrow down to a subset of “interesting” materials which would both guide and accelerate materials discover and lead to the next generation of complex nanomaterials.

Functionalization

When talking about functionalization, one of the most overlooked subjects is the actual ligand exchange or initial surface chemistry. Understandably, it is not an illustrious topic, as it is an intermediate enroute to any final product. It seems that in the magnetic nanoparticle community, everyone wants to have a nanoparticle with low size distribution, high crystallinity, etc. but they want it in an aqueous environment. Typically, these desirable characteristics are produced via the thermal decomposition synthesis, which is done using a hydrophobic ligand. It is only after some sort of encapsulation or ligand exchange that the particle become dispersible in aqueous media. There are many of these exchange procedures reported in literature, however they are difficult to repeat and often afford low yields. This is a highly pertinent issue that needs to be addressed as any sort of scale up requires multiple batches each with a yield of 2% or lower. This is not sustainable in the long run for any sort of biomedical application.
Disregarding the issues of ligand exchange, the possibilities of functionalization are almost endless when it comes to functionalization with biomolecules or other secondary functional moieties. Considering this, as well as the tunability of the particles, it is surprising that there is not more work in sensor applications. In vitro sensors are ideal from a technology perspective as they do not need to pass heavy FDA regulations and the working materials space is much wider as toxicity is of no concern. One could easily imagine a “cocktail” of particles each with a unique magnetic signature coupled with a unique biotag. This could be useful both for sensing low level metabolites, cell sorting, disease identification, selective color imaging, etc. Here the challenge would be the resolution of measurement. Considering the sensitivity of SQUIDs and the control over the nanomaterials that we have today, high resolution, high sensitivity measurements should be possible.

This does not take away from the fact that these materials are proving useful as possible in vivo treatments. As was seen in both chapter 3 and chapter 4, the potential as both a unique antibacterial platform, and as a targeted pharmaceutical agent is definitely there. It is just worrisome that after 30 years on magnetic nanoparticles there has been only one wide spread use (dextran coated, coprecipitation prepared, magnetite as a T2 contrast agent). It may be that in another 30 years we will see the in vivo use of nanomaterial based medicine, but adoption has been slow, even for relatively benign nanomaterials.

Application
Magnetic materials for use in medicine still has a ways to go before mainstream adoption. Many groups are currently working on trying to bring MagMED using magnetic particles into the clinical space. This is especially true in Europe where regulation is more reasonable when it comes to novel medicines. This MagMED treatment, demonstrate in chapter 3, is typically used as a cancer treatment and this is where almost all of the literature on MagMED is focused. There is however a lot of potential for it to be used as an alternative antibiotic for other much more devastating diseases as the glycan functional particles work two fold in preventing disease. Another area that could be interesting is using the gadolinium functionalized nanoparticles as ion sensors in MRI. Currently we are working on ion responsive gadolinium based contrast agents to measure calcium levels in vivo. In theory one of the larger issues with these agents will be that they are not ion specific. The gadolinium coated nanoparticles could be ion specific. The main difference would be that instead of using PEG as the stabilizing brush, a modified calmodulin protein could be used. In the presence of calcium 2+ the calmodulin protein undergoes a conformational change as the calcium binds. This would change the distance between the gadolinium on the outside of the particle and the magnetic core which would lower the R1. This change should be measurable in vivo using an MRI especially if optimal magnetite core and calmodulin length were used. This could work with any externally stimuli responsive polymers including pH, temperature, metabolite, etc.
Final Remarks

I do believe that magnetic materials will find adoption into clinical space. As nanoscientists we are lucky that there has already been precedence for a magnetic nanomaterial that has been FDA approved. I do believe that this will help bring the forefront of this field into the public space. I also believe that we have just scratched the surface of what these materials have to offer from a biomedical standpoint. Everything from diagnostics and imaging to disease treatment and prevention can be addressed through the intervention of nanomagnetics. Here is hoping that in my lifetime I get to see the small part I played make a difference in the future of heathcare.