MULTIFUNCTIONAL MAGNETIC NANOPARTICLES FOR CANCER IMAGING AND THERAPY

by

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*We also certify that written approval has been obtained for any proprietary material contained therein.
To my family,

Mike and Patty Westerfield, Karen Bennett, and Steve Foy,

for their unending encouragement and enthusiasm.
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LIST OF ABBREVIATIONS

Ab – Antibody
AUC – Area Under the Curve
BSA – Bovine Serum Albumin
CT – Computed Tomography
DI – Deionized
DOX – Doxorubicin
EDC – 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EPR – Enhanced Permeability and Retention
FTIR – Fourier Transform Infrared Spectroscopy
H$_2$O$_2$ – Hydrogen Peroxide
HAE – 4-hydroxyalkenals
HER2 – Human Epidermal Growth Factor Receptor 2
HLB – Hydrophilic Lipophilic Balance
K$_b$ – Binding Constant
MF – Magnetic Field
MCB – Mannitol Citrate Buffer
MDA – Malondialdehyde
MNPs – Magnetic Nanoparticles
MRI – Magnetic Resonance Imaging
NIR – Near Infrared
OA – Oleic Acid
‘O$_2^-$’ – Superoxide Anion
OH⁻ – Hydroxyl Anion
·OH – Hydroxyl Radical
PBS – Phosphate Buffered Saline
PEG – poly(ethylene glycol)
PEO – poly(ethylene oxide)
PI – Polydispersity Index
PPO – poly(propylene oxide)
PTX – Paclitaxel
PLA – poly(lactic acid)
PVA – poly(vinyl alcohol)
RES – Reticuloendothelial System
ROI – Region of Interest
ROS – Reactive Oxygen Species
SOD – Superoxide Dismutase
sulfo-NHS – N-hydroxysulfosuccinimide
Tfn – transferrin
Multifunctional Magnetic Nanoparticles for Cancer Imaging and Therapy

Abstract

by

SUSAN PATRICIA FOY

Tumor heterogeneity can significantly influence the efficacy of cancer chemotherapy and nanoparticle-mediated drug delivery. Therefore, there is a need of multifunctional nanoparticles that can deliver therapeutics and be tracked longitudinally. The research described in this thesis explores magnetic nanoparticles for applications in tumor identification, monitoring nanoparticle biodistribution, and cancer therapy. The multifunctional formulation can potentially be used in image-guided drug therapy to enhance diagnostic procedures and therapeutic outcomes.

The magnetic nanoparticle formulation consists of an iron-oxide magnetic core coated with oleic acid then stabilized with an amphiphilic block copolymer. Magnetic nanoparticles coated with the copolymer Pluronic F127 demonstrate sustained and enhanced magnetic resonance contrast in the whole tumor of a mouse breast xenograft tumor model. Hydrophobic near infrared dyes were loaded into the oleic acid coating of the magnetic nanoparticles to quantitatively determine their long-term biodistribution in vivo. The magnetic nanoparticles are visible in orthotopic breast tumors up to 12 days
post-injection through \textit{in vivo} fluorescence imaging. One-hour exposure to a magnetic field further enhances magnetic nanoparticle localization to the tumors.

The anticancer drugs doxorubicin and paclitaxel were loaded into the oleic acid layer of the magnetic nanoparticles for therapeutic treatment of breast cancer. Drug loaded magnetic nanoparticles effectively treat breast cancer cells \textit{in vitro}, while plain magnetic nanoparticles are non-toxic. Both plain and drug loaded magnetic nanoparticles significantly decrease tumor size and increase survival in tumor bearing mice compared to saline controls. Plain magnetic nanoparticles localized in the tumor as they breakdown may generate reactive oxygen species due to the Fenton reaction, causing apoptosis of cancer cells and leading to therapeutic effect without drug. The magnetic nanoparticles have the potential to be developed as an effective cancer theranostic agent, i.e., an agent with combined imaging and therapeutic applications.
CHAPTER 1: INTRODUCTION

1.1 Introduction

Over the past century, cancer prognosis has improved drastically through advances in diagnostic, surgical, and therapeutic treatments. However, due to the diverse array of cancers, we are still challenged with curing cancer. The difficulty in treating cancer is a result of both cancer heterogeneity and also challenges in selectively inhibiting cancer growth without affecting healthy tissues. Nanomedicine is a rapidly evolving field with aims of improving cancer outcomes by enhancing diagnostic procedures and therapeutic treatments. Nanomedicine refers to the design and application of nanoscale materials (one billionth of a meter), to improve human health and treat disease. Nanoparticles can entrap diagnostic and therapeutic agents, prolong their circulation time, increase their tumor localization and uptake, and decrease their systemic toxicity.

With goals of early cancer detection, targeted delivery of anti-cancer agents, and monitoring tumor response to therapy, nanoparticles are advancing from monofunctional particles to multifunctional agents that can be used to both diagnose and treat with the same nanoparticle formulation. There are many challenges in designing a nanoparticle for each individual application, and these challenges are compounded when trying to achieve each simultaneously. The challenges associated with nanoparticle delivery, and desirable characteristics of nanoparticles for in vivo use are discussed below. Strategies to maximize nanoparticle efficacy including multimodal, multifunctional, and targeted
therapeutics are evaluated. Taken together, we consider the features desired for multifunctional purposes and detail the design a magnetic nanoparticle with applications in cancer diagnosis, tumor targeting, and treatment.

1.2 General Strategies and Desirable Characteristics Required for Nanoparticles

One of the benefits of nanoparticles is the ability to ‘protect’ their payload from rapid degradation or clearance. By shielding drug, nanoparticles alter the absorption, distribution, metabolism, and excretion (pharmacokinetics) and effects of drug on the body such as toxicity (pharmacodynamics), compared to free drug. Nanoparticles can significantly prolong the circulation time of therapeutic agents leading to increased extravasation into tumors. Sustained release properties of nanoparticles can further lead to less frequent doses, lower toxicity, and better patient compliance, all of which directly affect clinical success. Four parameters that critically influence nanoparticle delivery are targeting, size and shape, charge, hydrophobicity of the nanoparticle. Each of these physical properties can be manipulated to optimize the in vivo behavior of nanoparticles and improve their diagnostic and therapeutic applications.

Nanoparticle targeting to tumors can be achieved passively thorough the enhanced permeability and retention (EPR) effect. This EPR effect results from a need for nutrients to support tumor growth forming a leaky vascular network within the tumor, and a corresponding ill-formed lymphatic drainage system compared to healthy tissue. The gaps and fenestrae in the tumor vasculature range from 100-600 nm in size, which allows nanoparticles with a maximum size of ~400 nm to extravasate into the tumor tissue.\(^1\)\(^2\)
The EPR effect is not always an effective targeting mechanism, however, because of the it’s dependency on the size of the nanoparticles and variability among tumors as certain tumors (e.g. pancreatic) are highly avascular. Other, more active forms of targeting are addressed below.

Organs of the reticuloendothelial system (RES) such as kidneys and liver can filter nanoparticles based on size. Nanoparticles < 10 nm in size can be filtered by the kidneys while the liver can rapidly capture particles greater than 100 nm. Nanoparticles are typically small and spherical in nature in order to achieve long circulation time and avoid rapid recognition and clearance by the RES. Shape of the nanoparticle beyond the basic spherical dimension may prolong circulation and increase uptake and retention in tumors as demonstrated by the unique wormlike structure of filomicelles that have a diameter of less than 100 nm but a length of up to 8 µm. Thus, while 100 nm is considered near optimal in size to prevent clearance by the RES and less than 400 nm to utilize EPR targeting, there are exceptions.

Apart from size and shape, nanoparticle surface coating and charge can also significantly alter how rapidly nanoparticles are cleared. Once injected, nanoparticles immediately interact with proteins in the blood stream. These proteins form a ‘corona’ around the nanoparticle and can contribute to the overall fate of the nanoparticles. Certain proteins that form the protein corona are known as opsonins (immunoglobulins, complement factors, and proteins such as fibrinogen, fibronectin, and lectin) and essentially mark the nanoparticles to be phagocytosed and cleared by the RES.
Interestingly, neutral particles may be opsonized and phagocytosed more slowly than positively charged particles in vitro, while dysopsonins such as albumin prevent RES clearance of the particles and prolong their circulation in the blood.

One final design characteristic of nanoparticles contributing to their prolonged blood circulation time is their coating. The surface coating can render otherwise hydrophobic drugs and particles dispersible in aqueous solution, protecting a nanoparticle that would be rapidly cleared and contribute to their stealth nature. One common coating is poly(ethylene) glycol (PEG); ‘PEGylation’ provides steric stabilization of the nanoparticles with a hydrophilic network that attracts water to the particle surface and further prevents opsonin binding, prolongs blood residence time, and prevents clearance by the RES. PEG also has very low toxicity and is currently approved by the FDA for clinical use, making it a popular choice for nanoparticle coating.

Nanoparticle formulations are synthesized with a variety of materials (carbon, gold, zinc, iron, gadolinium, lipids, and various polymers) to enhance tumor contrast, deliver drug, or ablate tumors. The biocompatibility of such materials, particularly those used as implantable devices is well studied, and the goal is for the medical devices to improve human health but not cause harm with long term exposure. Scaling objects down to the nanometer size range presents new challenges and toxicities not previously observed; the properties of such nanomaterials are often altered due to an increased surface area to volume ratio. The inverse relationship between size and cytotoxicity may increase the oxidative stress within cells and increase the production of
proinflammatory cytokines.\textsuperscript{22, 28} The size dependent properties of nanomaterials are often advantageous for cancer therapy, however, as smaller particles may produce responses not typically observed with their bulk counterparts such as generation of fluorescence or heat.\textsuperscript{22} Nanomaterials for cancer therapy may also be delivered as multiple doses, and it is essential to determine how these particles are degraded and cleared, and what types of long-term residual effects they may induce.

As a general summary, nanoparticles that can prolong blood residence time are typically less than 400 nm in size to extravasate into tumors, neutral or negative in charge to avoid opsonization, and have a hydrophilic PEG or other brush-like surface coating to contribute to their stealth nature and avoid rapid clearance by the RES. Each of these properties contributes to the overall efficacy of nanoparticles by increasing the amount of particles that can reach the tumor. As a result, there is increased local (tumor) contrast or drug delivery, decreased systemic toxicity, and the potential for less frequent dosing requirements and improved patient compliance. Proper dosing and prolonged or targeted drug delivery can further improve efficacy by preventing or overcoming drug resistance associated with current chemotherapeutics.

1.3 Nanoparticles for Imaging

Nanomaterials for diagnostic purposes are among the more successful nanoparticles to be translated to successful clinical applications.\textsuperscript{29-30} Healthy and pathologic tissue are not always easy to distinguish with traditional imaging modalities, and contrast agents are used to better define these differences. Iron-oxide nanoparticles
provide contrast to detect tumors for magnetic resonance imaging (MRI). Encapsulated iodine enhances computed tomography (CT), and gas encapsulated by a liposomal microparticle or polymer provides strong ecogenic properties for ultrasound contrast.\textsuperscript{21} Optical imaging of particles containing fluorophores or quantum dots provides potential to rapidly assess particle biodistribution and therapy response in small animal models, and is being explored to aid in surgical identification of tumor margins and to image whole breast tissue.\textsuperscript{31-33}

Nanoparticles offer the possibility to enhance cancer diagnosis, while the intrinsic nature of the nanoparticle and contrast agent can further enhance therapy.\textsuperscript{34} For example, endohedral metallofullerenol nanoparticles were originally designed as a tumor contrast agent.\textsuperscript{20,35} However, it was discovered that the fullerene cage encapsulating the contrast agent gadolinium could scavenge free radicals leading to indirect tumor ablation in mouse hepatomas. It is also suggested that iron-oxide nanoparticles, also designed as contrast agents and for magnetic hyperthermia, may act \textit{via} the opposite mechanism: generating additional oxidative stress through the Fenton reaction and inhibiting tumor growth.\textsuperscript{35-37} Hyperthermia of tumors containing these nanoparticles can further raise the local temperature to selectively kill cancer cells.

1.4 Nanoparticles for Therapy

1.4.1 Chemotherapy

Chemotherapy has significantly advanced the treatment of cancer over the past half-century, greatly increasing patient survival. Unfortunately, many of the original
challenges of conventional chemotherapy persist today including toxicity to healthy tissues, poor bioavailability as a result of hepatic or renal clearance, limited drug solubility that precludes drug administration via the intravenous route, and lack of dose-response effect. Additionally, concern of the chemotherapeutic side effects including alopecia and emesis may result in decreased patient compliance.

A major challenge in the design of novel chemotherapeutic agents to target cancer is their low solubility. Depending on the therapeutic approach used to design potential drug candidates, anywhere from 40-90 % of agents have poor water solubility which contributes to poor stability and low bioavailability.38 Agents must first be modified – which may alter their chemotherapeutic efficacy or negatively impact their bioavailability – or formulated with solvents so that they can be successfully administered. A major advantage to drug therapy then, is a focus on improving the intravenous delivery of these drugs with the aid of solvents and nanoparticles.

1.4.2 Nanoparticles as Drug Delivery Vehicles

Nanoparticles offer a variety of mechanisms to both carry and release their drug payload. Chemotherapeutic agents can be entrapped within a nanoparticle shell or matrix, or conjugated to the surface or internally in the nanoparticle.39-41 Each of these platforms presents a unique mechanism of drug release through diffusion of the drug from the nanoparticle to the surrounding environment, degradation of the nanoparticle leading to sustained drug release, or cleaving the bond between the drug and nanoparticle. Due to the variety of mechanisms of drug release, each of these formulations can further be
'tuned' such that drug is released at the site of the tumor through either a change in the environmental pH, or in response to an external stimulus such as heat or ultrasound that leads to more rapid breakdown of the nanoparticle and release of the drug payload.\textsuperscript{40, 42}

Despite the advantages of nanomaterials as drug delivery vehicles for cancer therapy, there are relatively few nanotherapeutics that are approved for clinical use. Only a handful of nanoparticle based therapeutics are currently available on the US market.\textsuperscript{19, 43} A review of the ongoing clinical trials reveals that there are nearly 100 trials involving nanoparticles and more than 500 trials based on liposomal formulations, most for the treatment of cancer (www.clinicaltrials.gov). Two nanotherapeutics to make it through clinical trials and be approved by the FDA for cancer therapy are a liposomal formulation of doxorubicin (Doxil) and a nanoparticle–albumin bound formulation of the drug paclitaxel (Abraxane).

Doxil was the first liposomal formulation to encapsulate anticancer agents approved by the FDA in 1995. Doxil greatly reduces the cardiotoxicity associated with free doxorubicin, but the liposomal formulation can preferentially accumulate in the palms and souls of the hands and feet. Such leakage results in redness, tenderness, and painful skin peeling for patients. Thus, while Doxil greatly reduces the cardiotoxicity associated with free doxorubicin, complications limit its complete substitution of the free drug Doxorubicin Hydrochloride. A PEG free liposomal formulation of doxorubicin known as Myocet does not have appreciable hand-foot syndrome like Doxil, and is approved in Europe and Canada for the treatment of metastatic breast cancer in
combination with cyclophosphamide.\textsuperscript{44-45}

Abraxane is a protein-stabilized nanoparticle formulation that encapsulates the mitotic inhibitor paclitaxel, and is the first FDA approved protein-stabilized nanoparticle formulation. This \textasciitilde{}130 nm particle consists of nanoparticle albumin bound paclitaxel, and eliminates the need for Cremophor EL as a solubilizing agent for the delivery of paclitaxel (Taxol). Cremophor EL presents challenges as a solvent for drug delivery due to hypersensitivity reactions and in extreme cases anaphylaxis and death.\textsuperscript{46} The advantages in the Abraxane formulation over Taxol include no hypersensitivity, a reduced infusion time from 3 h to approximately 30 min, higher administered doses with comparable toxicity, and improved efficacy against metastatic breast cancer in a Phase III clinical trial.\textsuperscript{47} Abraxane is also approved by the FDA for the treatment of metastatic breast cancer where other combination therapies have failed.\textsuperscript{48}

\textbf{1.4.3 Ligand Targeted Chemotherapeutics}

Targeting the over-expressed receptors on cancer as a mechanism to enhance therapy and prevent non-specific toxicity can be achieved through conjugating the drug of interest to an antibody, protein, or peptide. The targeting ligand may be exclusively expressed on the cancer cell such as HER2 (Human Epidermal growth factor Receptor 2) or over-expressed on cancer cells compared to normal cells (such as Transferrin) where the rapid cell growth leads to a rapid proliferation and a high demand for nutrients leading to the over-expression of various receptors. Ligand targeted therapeutics are an example of a successful application of this targeting strategy, with over 100 candidates
moving to clinical trials. In this instance, the drug of interest is conjugated directly to the targeting ligand without the use of nanoparticles, but there are limitations. For example, because of the low number of drug molecules that can bind to an antibody or antibody fragment (1-10) the drug must be highly potent, and a large amount must bind to be effective without saturating the binding sites. For these reasons, nanoparticles are a desirable choice to actively target a high-payload of drugs to the tumor. Nanoparticles can increase the drug delivered to thousands of drug molecules per targeting ligand as opposed to as many as 10 directly bound to the targeting ligand.

1.5 Challenges Surrounding Current Nanoparticle Therapies

A variety of monofunctional approaches to improve cancer diagnosis and therapies have been tested, but each has associated challenges that have limited their widespread applications. Nanoparticles that are based solely on the EPR effect may not be as effectively taken up by tumors as it was initially hypothesized. As demonstrated by Doxil, the liposomal formulations of doxorubicin leads to greatly reduced cardiotoxic side effects, but there is not necessarily improved efficacy. New challenges associated with altered tissue accumulation of the drug also arose. Like ligand-targeted therapeutics, optical imaging is being tested to guide surgical tumor resection, by conjugating fluorophores to cancer specific targeting moieties. Targeting strategies for both chemotherapeutics and fluorophores suffer from a low ratio of drug or fluorophores per targeting molecule.
1.6 Multifunctional Nanoparticles

Multifunctional nanoparticles offer a new strategy to more selectively target therapy to cancer and monitor the outcome. A nanoparticle containing a high payload of drug or contrast agent can be decorated with targeting ligands, offering an additional targeting mechanism beyond the EPR effect. This further enhances the amount of drug delivered beyond that achieved though basic ligand targeted chemotherapeutics. In addition, nanoparticles can be conjugated to several different targeting molecules producing a multivalent approach to tumor targeting.

Multimodal particles, i.e. particles that have dual function imaging applications, exploit complementary imaging techniques to further optimize diagnosis and provide accurate therapeutic intervention. For example, the high spatial resolution of MRI combined with highly sensitive optical imaging provide molecular information about the tissues with excellent anatomic location. With the introduction of surgical instruments and microscopes that allow for OI during surgery, multimodal nanoparticles with dual imaging applications can be used for both pre-operative planning and intra-operative staging.

There are many of multifunctional nanoparticles in development that employ a variety of strategies to improve therapeutic efficacy (Figure 1.1, page 12). Gold, single walled carbon nanotubes, and iron-oxide nanoparticles can all be used to enhance thermal ablation of a tumor with application of radiofrequency, microwave, or an alternating magnetic field. Hyperthermia can further sensitize cells increasing the therapeutic
Figure 1.1 Nanoparticle strategies for tumor ablation.
Nanoparticles containing drug or dye (1) are intravenously injected and extravasate into tumor tissue through the leaky vasculature by the EPR effect (2). Photoirradiation or hyperthermia (3) generates singlet oxygen or facilitates drug release from nanoparticles within the tumor (4) leading to tumor apoptosis and necrosis (5).

Figure adapted with permission from Manthe, et al. Copyright 2010 American Chemical Society.
effect of the drug, though this effect can be dependent on the order of heat generation and drug delivery. Quantum dots and photosensitizers can generate singlet oxygen upon excitation to cause tumor apoptosis and ablation. Nanoparticles consisting of temperature responsive polymers can selectively release their payload at the tumor upon stimulation, increasing drug only at the tumor and decreasing systemic toxicity. The various stimulus responsive therapies enhance the selectivity because both nanoparticles, and the stimulus are required simultaneously to achieve cell death. Multifunctional nanoparticles can also simultaneously entrap drug and contrast agents within a single formulation. The idea behind this ‘theranostic agent’ is that it can report drug location, and potentially be used to monitor an individual’s response to therapy long-term. Targeting strategies discussed below further enhance the delivery of each of these nanoparticles to limit systemic toxicity and enhance tumor therapy.

1.7 Targeting Nanoparticles to Tumors

The initial strategy of nanoparticle targeting involved the EPR effect, or the simple passive accumulation of nanoparticles based on size into the leaky tumor vasculature. Two additional targeting strategies can increase the accumulation of nanoparticles injected intravenously at the tumor. The first, discussed above, involves attaching ligands to the nanoparticles to increase their retention or internalization by cancer cells. This ligand targeting approach does not always increase the amount of nanoparticles that accumulate in the tumor compared to non-targeted nanoparticles because both initially reach the tumor passively. However, once localized to the tumors, receptor targeted particles improve efficacy due to receptor-mediated internalization and
intracellular drug release. The choice of ligand also alters the therapeutic response. While internalizing antibodies provide better efficacy, non-internalizing antibodies that allow the nanoparticle to remain at the cell surface may promote bystander effects allowing drug to enter surrounding cancer cells not expressing the targeting ligand.\textsuperscript{49,54}

Ligand targeting strategies can be further improved by targeting receptors expressed on the tumor vasculature in the absence of or in combination with receptors expressed on the tumor cells themselves.\textsuperscript{3} The advantage of this strategy is that nanoparticles may be more likely to come into contact with these vascular. The second approach of drug targeting involves attracting particles with magnetic properties to the tumor by applying an external magnetic field. This approach increases the nanoparticle accumulation and efficacy compared to non-targeting, but it is limited to superficial tumors.\textsuperscript{55}

1.8 Challenges and Limitations

One challenge in creating a multifunctional nanoparticle is optimizing each application (imaging, therapy, targeting) without compromising the efficacy of the other functions. In creating a multimodal imaging device that combines optical and magnetic resonance imaging, the iron oxide can interfere with the fluorescence, decreasing optical sensitivity.\textsuperscript{56-57} Dual encapsulation of therapeutic and imaging agents within particles may inhibit the imaging properties or reduce the amount of drug loading. When therapeutic and targeting agents are conjugated to the surface, the agents compete for the same, limited number of binding sites, and the conjugation may alter the effectiveness of the drug or targeting ligand, or alter the properties and biodistribution of the particles as the moieties are cleaved.
Many of the nanoparticle formulations for drug delivery involve chemical conjugation or ionic binding of the drug to the surface polymer of the nanoparticle.\textsuperscript{58} Such formulations show limited drug loading and release of the drug in a few hours.\textsuperscript{58} Thus, it may be advantageous for the drug and imaging agents to be entrapped within the nanoparticle both to prevent altering the surface properties, and to allow the drug to be released at the site of the tumor.

1.9 A Multifunctional Magnetic Nanoparticle for Cancer Diagnosis and Therapy

To address the challenges associated with monofunctional nanoparticle formulations, we have developed a multifunctional magnetic nanoparticle (MNP) with the goal of therapeutic, targeting, and imaging applications in a single formulation. The goal was to integrate the features and balance the applications without compromising the effectiveness of each individual property. Our MNP consists of an iron-oxide core coated with Oleic Acid (OA) and stabilized with the tri-block copolymer Pluronic F127. Hydrophobic drugs or near infrared (NIR) dyes are loaded into the OA layer of the MNP by diffusion, and are not chemically conjugated to the nanoparticle surface (\textit{Figure 1.2}, page 16). This loading method allows high drug loading efficiency without altering the surface properties such as size and charge that would then alter biodistribution. The MNPs release the drug over a period of weeks helping to achieve optimal dosing by reducing systemic toxicity, and decreasing the likelihood that drug resistance would develop from insufficient drug present.\textsuperscript{39,59} The surface remains free for conjugation to tumor targeting ligands, and thus active uptake of the nanoparticles by the tumor via targeting ligands or active targeting of the MNPs to the tumor via magnetic field (MF)
Figure 1.2 Schematic of a magnetic nanoparticle.

A magnetic nanoparticle with hydrophobic drug or fluorescent NIR dye loaded in the hydrophobic oleic acid layer.
targeting can further increase MNP localization to the tumor.

Currently, the magnetic properties generate contrast for MRI. Optical imaging provides a complementary imaging platform that is less expensive in pre-clinical testing, allows the nanoparticles to be tracked for long-term biodistribution, and is sensitive and quantitative. Clinically, NIR dyes as a secondary imaging agent will be particularly advantageous in breast cancer, as the NIR dyes can penetrate ~ 10 cm through tissue, an ideal penetration depth for breast cancer imaging. Additionally, while MRI provides contrast in the diagnostic setting, optical imaging technologies enable surgeons to view in real time the fluorescence signal from nanoparticles at the tumor margins aiding in tumor identification and tumor resection.

1.10 Hypothesis

We hypothesize that our multifunctional magnetic nanoparticle, with applications in imaging and drug therapy, will enhance tumor identification, targeted drug delivery, and tumor response to therapy.

1.11 Project Goals/Specific Aims

Specific Aim 1: Design, synthesis, and characterization of magnetic nanoparticles for combined drug delivery and imaging applications.

Specific Aim 2: Evaluate magnetic nanoparticles as a platform for magnetic resonance imaging and optical imaging and the effect of external magnetic field targeting on tumor localization of magnetic nanoparticles.
**Specific Aim 3:** Evaluate efficacy of magnetic nanoparticles in treating breast tumors.

1.12 General Overview of the Dissertation

This dissertation is divided into chapters based loosely on the specific aims described above. An introduction to the methods for MNP formulation and synthesis, optimization and characterization is first given in Chapter 2 (Aim 1). Chapters 3 and 4 focus on *in vivo* MRI and optical imaging with an application demonstrating the effects of MF targeting (Aim 2). Chapter 5 details the *in vivo* tumor response to MNP therapy corresponding to Specific Aim 3. A discussion on the potential applications of MNPs for cancer treatment by generating oxidative stress and a concluding summary is given in Chapter 6.
CHAPTER 2: MAGNETIC NANOPARTICLE SYNTHESIS

2.1 Introduction

The magnetic nanoparticle platform detailed in this work is both multimodal and multifunctional with applications in magnetic resonance and optical imaging, targeting, and drug delivery. The MNP formulation consists of an iron-oxide magnetic core, which is coated with oleic acid (OA) and then stabilized with a block copolymer, rendering the formulation dispersible in water and suitable for intravenous administration. This chapter describes the synthesis of the MNPs with optimization of the surface coating, details drug and dye loading, and outlines a conjugation method for targeted cancer delivery.

Nanoparticle characteristics such as size, charge, dispersion stability, and macrophage uptake greatly influence the systemic circulation time of MNPs and their localization efficiency in the tumor. Thus we first examined the effects of different pluronic and tetronic block copolymers as surface coatings on the characteristics of MNPs. Different pluronics and tetronics vary structurally, as well as in the ratio of hydrophobic PPO and hydrophilic PEO segments in the polymer chain and in molecular weight (Table 2.1, page 20). The pluronic block copolymer consists of a hydrophobic poly(propylene oxide) (PPO) chain flanked on each side with hydrophilic poly(ethylene oxide) (PEO) chains. Tetronic block copolymers contain four PPO–PEO chains connected together at the PPO subunits to form a star shape structure (Figure 2.1, page 21). We hypothesized that how well these block copolymers anchored to the OA coating
Table 2.1 Properties of Pluronic and Tetronic Block Copolymers Used to Coat MNPs

<table>
<thead>
<tr>
<th>Co-polymer</th>
<th>Formula</th>
<th>% PEO</th>
<th>Molecular Weight</th>
<th>HLB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feridex IV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pluronic F68</td>
<td>H(EO)<em>{76}(PO)</em>{29}(EO)_{76}</td>
<td>80</td>
<td>1680</td>
<td>6720</td>
</tr>
<tr>
<td>Pluronic F108</td>
<td>H(EO)<em>{133}(PO)</em>{50}(EO)_{133}</td>
<td>80</td>
<td>2920</td>
<td>11680</td>
</tr>
<tr>
<td>Pluronic F127</td>
<td>H(EO)<em>{100}(PO)</em>{60}(EO)_{100}</td>
<td>70</td>
<td>3780</td>
<td>8820</td>
</tr>
<tr>
<td>Pluronic L64</td>
<td>H(EO)<em>{13}(PO)</em>{30}(EO)_{13}</td>
<td>40</td>
<td>1740</td>
<td>1160</td>
</tr>
<tr>
<td>Tetronic 904</td>
<td>[-CH2N((PO)<em>{17}(EO)</em>{13}H)]_{2}</td>
<td>40</td>
<td>4020</td>
<td>2680</td>
</tr>
<tr>
<td>Tetronic 908</td>
<td>[-CH2N((PO)<em>{22}(EO)</em>{114}H)]_{2}</td>
<td>80</td>
<td>5000</td>
<td>20000</td>
</tr>
</tbody>
</table>

PEO, poly(ethylene oxide); PPO, poly(propylene oxide); HLB, hydrophilic-lipophilic balance. Information obtained from the BASF website.
Each particle contains an iron-oxide core coated with OA and is coated with either pluronic (single PEO-PPO-PEO subunit) or tetronic (two PEO-PPO-PEO subunits). The PPO subunit from the copolymers adsorbs onto the OA rendering the MPs dispersible in aqueous solution (Pluronic and tetronic are registered trademarks of BASF SE, Ludwigshafen, Germany).
could significantly influence the properties of the MNPs (e.g., particle size, protein binding and binding constant, dispersion stability in phosphate-buffered saline [PBS], and macrophage uptake).

After optimizing the surface coating of the MNP formulation, we next tested the formulation for loading near-infrared dyes into the OA layer of the MNP. Loading these agents into the hydrophobic shell allows for both high loading efficiency (as opposed to conjugation to a limited number of surface sites) and prevents altering the surface properties of the nanoparticles. We have previously used this method for drug loading into the MNP allowing for sustained release over time.39

Finally, we detail a method for conjugation of transferrin to the MNP to further enhance uptake of the nanoparticles once localized to the breast tumors. Unlike the loading of drug and dye into the OA layer, transferrin is conjugated to the carboxyl group of the OA. By loading drug and dye into the nanoparticles through physical interactions between the hydrophobic layers, more sites are free for conjugation. Drug and dye are not competing for the same binding sites as transferrin, and complicated conjugation chemistry is not required. However, conjugation of transferrin may ultimately influence the surface properties and thus RES interaction and clearance in vivo may be different from the optimized plain MNP formulation.
2.2 Materials and Methods

2.2.1 Reagents

Pluronics (F127, F68, F108, and L64) and Tetronics (T904 and T908) were a gift from BASF Corporation (Mt. Olive, NJ). Feridex IV was purchased from Berlex Laboratories (Montville, NJ). Hydrophobic NIR dyes (SDB5700, SDA5177, SDA2826, SDA6825, and SDB5491) were purchased from H.W. Sands Corporation (Jupiter, FL). We purchased ammonium hydroxide (5 M), EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), hydrochloric acid, iron(III) chloride hexahydrate (FeCl$_3$•6H$_2$O, pure granulated, 99%), iron(II) chloride tetrahydrate (FeCl$_2$•4H$_2$O, 99+%), N-hydroxysulfosuccinimide (sulfo-NHS), and oleic acid (OA) from Fisher Scientific (Pittsburgh, PA); FITC transferrin antibody from Abcam (Cambridge, MA); FITC Transferrin from human serum from Invitrogen (Carlsbad, CA), and triethylamine ($\geq 99.5\%$) from Sigma-Aldrich (Saint Louis, MO). Deionized water freshly purged with nitrogen gas was used as described below in all of the steps involved in MNP synthesis.

2.2.2 Magnetic Nanoparticle Synthesis and Characterization

The steps involved in magnetic nanoparticle synthesis, drug or NIR dye loading, and separation are shown in Figure 2.2, page 24.

2.2.2.1 Magnetic Nanoparticle Synthesis

Magnetite particles were synthesized by a co-precipitation reaction with Fe(II) and Fe(III) in the presence of ammonium hydroxide.$^{39}$ A 15-mL solution of 0.1 M Fe(II)
Figure 2.2 Magnetic nanoparticle synthesis.
Magnetic nanoparticles are synthesized via a co-precipitation reaction with Iron (II), Iron (III), and ammonium hydroxide. The magnetite core is then coated with Oleic Acid, and stabilized with a pluronic block co-polymer. Hydrophobic drug or NIR dyes are loaded into the Oleic Acid layer with overnight stirring. The MNPs are then washed and separated from free drug/dye with magnetic separation.

was combined with 30 mL of 0.1 M Fe(III) and stirred for 20 min under a fume hood. Ammonium hydroxide (3 mL, 5 M) was added drop wise to the solution to precipitate magnetite particles. OA (100 mg) was added to the magnetite particles, and the solution was heated to 80 °C for 30 min to evaporate off ammonia gas. The solution was cooled to room temperature, washed once by magnetic separation by placing a magnet (12,200 G, Edmund Scientific, Tonawanda, NY) below the beaker for 5 min and resuspended in 45 mL of water.

After washing the OA coated MNPs, particles were prepared with different pluronic and tetronic block copolymer coating. One hundred milligrams of each one of the block copolymers (F127, F68, F108, L64, T904, or T908) were added to OA-magnetite particles prepared as above, and the particle suspension was kept overnight under constant magnetic stirring. The MNP suspension was washed three times using magnetic separation and centrifuged at 1,000 rpm for 15 min at 10 °C to remove large aggregates. The ratio of OA to pluronic F127 was optimized in our previous studies to obtain small MNPs with a low polydispersity index.39 This amount of block copolymer is in excess of what can bind to the MNPs, so we used 100 mg of each of the different block copolymers for surface modification of the MNPs.

2.2.2.2 Magnetic Nanoparticle Characterization

Dynamic laser light scattering with the NICOMP 380 ZLS (Particle Sizing Systems, Santa Barbara, CA) was used to measure the mean hydrodynamic particle size of the MNPs, comparing different pluronic or tetronic coatings in water, PBS, 0.1%
bovine serum albumin (BSA, Fraction V, Sigma–Aldrich, St. Louis, MO) in water, and mannitol–citrate buffer, pH 7.4. The size of the iron-oxide core of the MNPs was determined using a Philips 201 transmission electron microscope (Philips/FEI Inc., Briarcliff, Manor, NY). Samples were heated at the rate of 20 °C/min under 20 mL/min nitrogen gas flow to obtain a thermogram for each sample between 30 °C and 1000 °C. Lyophilized samples were used to confirm the adsorption of the pluronic and tetronic block copolymer coatings with a Fourier transform infrared (FTIR) spectrometer (PerkinElmer LLC, Shelton, CT), and to determine the OA and pluronic or tetronic content of the MNPs by thermogravimetric analysis (Pyris 1 TGA, PerkinElmer LLC, Shelton, CT). A 1 mL sample of MNPs was lyophilized for 24 h and weighed to determine the MNP yield. The iron content of the MNPs was estimated using the 1,10 phenanthroline colorimetric method.

2.2.3 Magnetic Nanoparticle Synthesis for Drug/Dye Loading

2.2.3.1 Conversion of DOX•HCl into Water-insoluble Doxorubicin

A 49 mg sample of DOX•HCl was weighed in a small beaker and 14 mL of 12.5% v/v methanol in chloroform was added to the sample and briefly sonicated in a water bath style sonicator. Sixty microliters of triethylamine was added to the above solution and stirred for 2-3 h. The solution was filtered into a 20 mL vial (of known mass) with a 10 mL syringe and 0.02 mm filter (Inorganic membrane syringe driven filter, Anatop 25; Whatman International Ltd, Maidstone, England), covered with aluminum foil with holes in the top and left in a fume hood to begin evaporation. The sample was lyophilized to remove residual chloroform, dry weight determined, and
stored protected from light at -20 °C.

2.2.3.2 Doxorubicin in Solution

A 4 mg/mL concentrated solution of hydrophilic DOX was prepared by dissolving DOX•HCl into a 66% v/v solution of ethanol in sterile water with vortexing. The solution was stored protected from light at -20 °C and diluted in media for in vitro experiments.

2.2.3.3 Drug Loading in MNPs

Hydrophobic DOX was suspended at 5 mg/mL in ethanol, sonicated briefly, and 600 µL of the DOX solution was added drop wise while stirring to 30 mg MNPs suspended in 7 mL of water in a 20 mL vial. The solution was stirred overnight, and the drug incorporated into the OA shell surrounding the MNPs. Unentrapped drug was separated from the MNPs by magnetic separation; the MNPs were washed three times, resuspending the particles in water and saving the supernatant.

One milliliter samples of the supernatant were centrifuged for 10 min at 14,000 rpm in an Eppendorf microcentrifuge to remove any particles, and the supernatant analyzed in a fluorescence spectrophotometer at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 591$ nm. Standards of 0-10 µg/mL in water were used to determine the concentration of drug in the wash. The drug loading in MNPs was determined by subtracting the drug in the wash from the total amount of drug added to the MNPs.
2.2.3.4 NIR Dye Loading in MNPs

MNPs (30 mg in 7 mL water) were combined with 600 µL of 0.25-5.0% w/w NIR dye in ethanol with magnetic stirring. The solution was stirred overnight to allow the dyes to partition into the OA layer of the MNPs. The dye-MNPs were then washed three times by magnetic separation, and the washings were saved for analysis. The washings were centrifuged at 13,000 rpm to remove MNPs, and the supernatant collected. A sample of the supernatant was diluted in ethanol, and the dye’s peak absorbance was measured on a UV/VIS spectrophotometer (DU 640B; Beckman Coulter, Brea, CA). Stock solutions (1 mg/mL) of each dye were prepared in ethanol and dilutions were made in ethanol (0-10 µg/mL). Dye loading in MNPs was determined by subtracting the concentration of dye in the supernatant from the total amount of dye added.

2.2.4 Transferrin Conjugation to MNPs

2.2.4.1 Transferrin Conjugation with sulfo-NHS and EDC

An amide bond was formed between the amine group of the FITC transferrin and the carboxyl group on the OA layer of the F127 MNPs. 10 mg of F127 MNPs were placed in an 8 mL glass vial, separated with a magnet, and suspended in a total volume of 3 mL water. FITC Transferrin (50 µL, 250 µg) was added to the F127 MNPs and gently stirred on a magnetic stir plate. 250 µL EDC in PBS and 250 µL Sulfo-NHS in PBS were added to the reaction so that the final concentrations were 0.1 M and 5 mM respectively. The reaction was stirred for 2 h at room temperature. F127 MNPs conjugated to the transferrin (Tfn-MNPs) were then separated from free transferrin, EDC, and sulfo-NHS by magnetic separation. The particles were washed three times with water, and the
supernatant saved from each wash. A sample of the supernatant from each wash was spun
down at 13,000 rpm for 10 min to remove any remaining MNPs, and the resulting
supernatant analyzed for free FITC Transferrin with a PerkinElmer LS55 Fluorescence
spectrophotometer in a white 96-well plate (Ex=480 nm, Em=530 nm). Standards of
FITC transferrin antibody were prepared in the wash of plain MNPs from 0-8 µg/mL.
The percentage of transferrin conjugation to the F127 MNPs was calculated by
subtracting the amount of free transferrin in the washes from the total amount of
transferrin added.

2.2.4.2 Transferrin Conjugation with EDC and Glycine

In subsequent experiments conjugation without sulfo-NHS and with the addition
of glycine to quench the reaction were attempted to prevent non-specific conjugation and
decrease the aggregation caused by the addition of sulfo-NHS. The conjugation occurred
as described above with the following modifications. After addition of EDC,
nanoparticles were stirred on a magnetic stir plate for 0.5 – 2.0 h. A 250 µL solution of
glycine (2 M for final concentration of 0.15 M) was then added to the reaction and stirred
for an additional 0.5 – 2.0 h. Conjugation efficiency was determined as described above,
by analyzing the amount on un-bound Transferrin in the wash and comparing it to
standards prepared in the wash of MNPs without Transferrin.

2.2.4.3 Transferrin Antibody Binding to MNPs

To confirm that Transferrin was conjugated to the MNPs and was functional,
Transferrin Antibody (Tfn-Ab) was added to the MNPs and the amount bound to the
MNPs calculated. Samples of plain F127 MNPs or Tfn-MNPs (1 mL, 1 mg MNPs/mL) were suspended in microcentrifuge tubes and separated with a magnet from the wash for 30 min. This pre-treatment wash was saved to create standards. A stock solution of 20 µg/mL FITC Tfn-Ab in water was diluted from 0-20 µg/mL and added to the MNP pellets. The microcentrifuge tubes were left on a rotating shaker at room temperature for 2 h protected from light. MNPs were separated with a magnet 2 × and the washes collected. The washes (and standards of 0 – 20 µg/mL created in the pre-treatment wash) were centrifuged at 13,000 rpm for 10 min at 4 ºC. The supernatant was collected and the amount of FITC fluorescence measured in a white 96 well plate with a PerkinElmer LS55 Spectrophotometer (Ex 480 nm, Em 530 nm). The amount of fluorescence detected in the wash was used to calculate the total amount of Tfn-Ab bound to the MNPs.

2.3 Results

2.3.1 Magnetic Nanoparticle Characterization

We examined the properties of different pluronic and tetronic block copolymers as surface coatings for our MNPs. The FTIR spectra of each MNP formulation confirmed anchoring of the different types of pluronic and tetronic onto the OA-coated iron-oxide core. The block copolymers exhibited broad FTIR bands within the range 1250 cm⁻¹–1000 cm⁻¹ due to C–O–C stretching and CH₂ rocking vibrations in the PEO/PPO chains (Figure 2.3 A, page 31). The magnetic cores of the MNPs did not produce a signal in this range. In the FTIR spectra, the characteristic C–O–C and CH₂ peaks confirm the adsorption of each block copolymer to the MNP surface (Figure 2.3 B, page 31).
Figure 2.3 FTIR of various pluronic and tetronic coatings on magnetite particles.

A) FTIR peaks characteristic for pluronic F127 are present in F127-modified MNPs and not OA-magnetite core confirming adsorption of the pluronic to the magnetite core. B) Peaks for the C-O-C and CH2 functional groups of pluronic and tetronic copolymers confirm that each of the six copolymers anchors onto the magnetite particle.
We determined the OA and pluronic/tetronic block copolymer content for each MNP formulation by thermogravimetric analysis. Controls included bare MNPs and OA-coated MNPs. Mass loss for all formulations occurred between 200 °C and 400 °C, with the inflection point ranging from 280 °C to 400 °C. The particle composition was predominantly iron-oxide for each formulation (70.1–78.0 wt%) depending on the copolymer bound, and the OA content ranged from 15.4–17.1 wt%. The copolymer adsorption ranged from 4.9 wt% (L64-modified MNPs) to 14.5 wt% (F127-modified MNPs, Table 2.2, page 33).

Generally, as the MW of the PPO increased, a greater percent of copolymer was able to bind to the MNPs. This correlation considers the structure of the PPO (Figure 2.1, page 21) as copolymers anchor into the OA. The pluronic copolymers (L64, F68, F108, and F127) fold in half to anchor onto the OA; the depth at which the PPO chain can anchor onto the OA is at most one half the overall length of the PPO (Figure 2.1, page 21). Tetronic T904 and T908 have greater PPO molecular weight than any pluronic (Table 2.1, page 20); however, the copolymer is branched, forming a star shape, allowing tetronic to anchor into the OA a depth of at most one fourth of the overall linear length of the PPO chain (Figure 2.1). The PPO chain strengthens the copolymer interaction with the OA-coated MNP, allowing more copolymer to bind and stay bound during MNP synthesis. If the PPO anchoring is inadequate, more of the hydrophobic OA-magnetite core is exposed and the MNPs aggregate.

Transmission electron micrographs were similar for all six formulations, with the
Table 2.2 Effect of Pluronic and Tetronic Coatings on Magnetic Nanoparticle Characteristics

<table>
<thead>
<tr>
<th>Co-polymer</th>
<th>% Co-polymer bound to MNPs</th>
<th>Diameter (nm) and (Polydispersity Index)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DI water</td>
</tr>
<tr>
<td>Feridex IV</td>
<td>-</td>
<td>143 (0.29)</td>
</tr>
<tr>
<td>Pluronic F68</td>
<td>6.0</td>
<td>216 (0.12)</td>
</tr>
<tr>
<td>Pluronic F108</td>
<td>8.6</td>
<td>206 (0.09)</td>
</tr>
<tr>
<td>Pluronic F127</td>
<td>14.5</td>
<td>194 (0.10)</td>
</tr>
<tr>
<td>Pluronic L64</td>
<td>4.9</td>
<td>186 (0.10)</td>
</tr>
<tr>
<td>Tetronic 904</td>
<td>8.5</td>
<td>196 (0.15)</td>
</tr>
<tr>
<td>Tetronic 908</td>
<td>7.5</td>
<td>228 (0.11)</td>
</tr>
</tbody>
</table>

MNPs, magnetic nanoparticles; DI, Deionized; MCB, mannitol citrate buffer; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

* Particle size measured following 4 h incubation in the presence of 100 µg/mL BSA in water. Particle size represents hydrodynamic diameter. Core diameter (measured by transmission electron microscopy) was 10-15 nm. MNPs have negative zeta potentials, typically ranging from -20 to -30 mV.
iron-oxide cores measuring ~10–15 nm. The suspending medium greatly influenced MNP size. In water, the hydrodynamic diameter of the MNPs ranged from ~185 to 230 nm for each of the formulations (Table 2.2, page 33). The mean hydrodynamic diameter of Feridex IV, a commercial contrast agent, was ~140 nm, either in water or PBS, which is smaller than the mean diameter of our MNPs. However, the polydispersity index of our MNPs is significantly lower than that of Feridex IV (polydispersity index = ~0.1 vs. 0.3), suggesting the heterogeneous nature of Feridex IV as compared to our MNPs. Tetronic T904- and pluronic F68- and L64-modified MNPs aggregated in PBS. The MNP size did not change in the presence of BSA. In general, mannitol–citrate buffer was found to be the suitable medium for suspending our MNPs because it did not increase the MNP size significantly compared to MNPs suspended in water.

Particles modified with F68, L64, and T904 had a significant increase in size when dispersed in PBS. The increase in size was likely due to the short PPO length available for anchoring, which resulted in low surface coating of these polymers, as confirmed by thermogravimetric analysis (Table 2.2, page 33). This loose anchoring of the above block copolymer may have resulted in their dissociation in the presence of PBS (salting out effect), causing particles to aggregate (Table 2.2). The overall results suggest a balance between PPO and PEO chain lengths is essential to anchor the block copolymer via the PPO chain onto MNPs, as well as for their hydration via the PEO chain to maintain a dispersed state.
2.3.2 NIR Dye Loading

To determine whether our MNPs could be used for optical imaging, we tested five commercially available NIR dyes in our MNP formulation and compared their stability, toxicity, and level of fluorescence. These dyes have not been tested for optical imaging for biomedical applications but have been used for applications in credit and security card technology and as inks for laser reading devices. However, the characteristics of the dyes, particularly their hydrophobic nature and NIR excitation and emission wavelengths, are considered suitable for incorporation in our MNPs for in vivo imaging. The absorbance curves for each dye when dissolved in ethanol and the emission spectra are shown in Figure 2.4 (page 36). Peak absorbance ranged from 683–775 nm (dyes 5700, and 5491) and peak emission ranged from 773–830 nm (dyes 5700 and 2826).

The hydrophobic dyes were each dissolved in ethanol and added to the MNPs with overnight stirring at concentrations between 0.25-5.0% w/w MNPs. The size, zeta, and percent loading of the dyes in the MNPs are listed in Table 2.3, page 37. The dyes partitioned into the OA layer of the MNP formulation with 100% loading efficiency for dyes 5700, 2826, and 5491 at all concentrations tested. Dye 5177 loaded into the MNPs at 58.2 to 73.3%. Dye 6825 loaded into the MNPs at 100% for the lowest four concentrations (0.25-2.0% w/w); however, at 5% w/w, dye 6825 did not partition into the MNPs because the MNPs had aggregated on the stir bar and could not be recovered. In general, the size of the nanoparticles was less than 300 nm after loading each of the dyes at each of the varying concentrations. The Zeta potential became slightly more positive with increasing dye concentration for MNPs loaded with dyes SDA 2826 and SDA 6825.
Figure 2.4 Absorbance and emission spectra of NIR dyes.
Five NIR dyes from H.W. Sands were tested for optical imaging of MNPs.
### Table 2.3 Magnetic Nanoparticle Characteristics after NIR Dye Loading

<table>
<thead>
<tr>
<th>Dye % w/w</th>
<th>SDB5700</th>
<th>SDA5177</th>
<th>SDA2826</th>
<th>SDA6825</th>
<th>SDB5491</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>227.1 (0.110)</td>
<td>253.1 (0.105)</td>
<td>245.2 (0.124)</td>
<td>255.4 (0.120)</td>
<td>266.4 (0.176)</td>
</tr>
<tr>
<td>0.25</td>
<td>262.7 (0.253)</td>
<td>248.4 (0.203)</td>
<td>227.4 (0.080)</td>
<td>257.5 (0.221)</td>
<td>214.0 (0.321)</td>
</tr>
<tr>
<td>0.5</td>
<td>319.1 (0.321)</td>
<td>220.8 (0.163)</td>
<td>219.4 (0.107)</td>
<td>219.3 (0.136)</td>
<td>267.4 (0.386)</td>
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<td>1</td>
<td>218.4 (0.125)</td>
<td>259.4 (0.144)</td>
<td>203.7 (0.099)</td>
<td>226.8 (0.118)</td>
<td>188.4 (0.238)</td>
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<th>SDA2826</th>
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<th>Dye % w/w</th>
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</table>
We have used this simple method to load hydrophobic anticancer agents for doxorubicin (base) and/or paclitaxel within the OA layer of our MNPs with high loading efficiency and sustained release over several weeks.\textsuperscript{39,60} Although the dye is not chemically conjugated to our MNPs, strong hydrophobic interactions with the OA layer of the MNPs trap the dye within the particles, preventing its rapid leaching (\textbf{Figure 1.2}, page 16).

The size and charge of the particles did not change with the addition of the NIR dyes at the concentrations tested. The hydrodynamic diameter of the MNPs was \textasciitilde240 nm as determined by dynamic light scattering. The zeta potential of plain and dye-loaded MNPs was about -30 mV. By avoiding chemically conjugating the dye to the surface, the surface properties were not altered by the charge of the dye, an important feature because the surface charge can alter the biodistribution profile of nanoparticles.\textsuperscript{63}

\textbf{2.3.3 Transferrin Conjugation}

We found that an amide bond could be formed between the carboxyl group of the oleic acid layer of our MNP formulation and an amine group of the target molecule – transferrin, with addition of sulfo-NHS and EDC. Sulfo-NHS, which stabilizes the EDC increased the rate of flocculation and aggregation of the MNPs under visual examination, and also led to an increase in particle size. This increase in particle size was especially apparent with the addition of glycine, used to quench the reaction (\textbf{Table 2.4}, page 39). In subsequent experiments, sulfo-NHS was excluded, and we observed a smaller particle size, less visual flocculation and aggregation, and a slightly lower conjugation efficiency (79 \% without sulfo-NHS vs 96\% with sulfo-NHS).
### Table 2.4 Transferrin Conjugation Conditions and Magnetic Nanoparticle Characteristics

<table>
<thead>
<tr>
<th>MNP Conjugation Conditions</th>
<th>MNP Characteristics</th>
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<td>651.6</td>
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<tr>
<td>X X X*</td>
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</tr>
<tr>
<td>X X</td>
<td>239.9</td>
</tr>
<tr>
<td>X X X X+</td>
<td>284.6</td>
</tr>
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</table>

* 90 min stirring
+ Gly = 1.0 mL, 2 M for 0.465 final concentration
We confirmed the amide bond formation by two methods. First, *via* FTIR, where peaks at ~1650 and ~1575 cm\(^{-1}\) are characteristic of the C-N and C=O stretching vibrations and N-H bending vibrations of the amide I and II bonds (Figure 2.5, page 41). Second, to confirm that Transferrin was bound and active, we added increasing amounts of Transferrin-Ab to the Tfn-MNPs (Figure 2.6, page 42). There was some non-specific interaction between Transferrin-Ab at higher concentrations and plain MNPs and also control MNPs in the presence of EDC. This interaction was reduced with the addition of Glycine, which was used to quench the reaction and prevent non-specific charge interactions. The binding of Tfn-Ab to the MNPs synthesized with EDC, Gly, and Tfn, but not to those synthesized without Tfn confirms the interaction is between the Tfn-Ab and Tfn, and not with the MNPs.

**2.4 Troubleshooting**

The following points on handling the MNPs should aid in the synthesis and prevent potential problems:

- If boiled during synthesis, MNPs will not suspend.
- When determining yield of a lyophilized sample of MNPs, nitrile and latex gloves produce static charge of the MNPs that may alter the measured yield. Handling the lyophilized sample with forceps or bare hands may reduce this problem.
- MNPs must be stored refrigerated (4 °C). Lyophilized or frozen (0 °C or below) samples will aggregate and will not resuspend even with vortexing or water bath sonication.
Figure 2.5 FTIR after transferrin conjugation to MNPs.

Presence of peaks at 1650 and 1575 cm$^{-1}$ in Transferrin-MNPs, but not plain F127 MNPs confirms amide bond between carboxyl group of Oleic acid on MNPs and amine group on Transferrin.
Figure 2.6 Transferrin conjugated MNPs bind transferrin antibody.

Transferrin Antibody (Tfn-Ab) binding confirms proper orientation of Transferrin. Tfn-Ab interacts non-specifically with MNPs synthesized with the cross-linker EDC either by conjugation or charge interaction. This interaction decreases in the presence of glycine (Gly), used to quench the reaction. An increase in Tfn-Ab binding to MNPs synthesized with Gly confirms Tfn-Ab binds to the Transferrin on the MNPs.
• MNPs must be suspended in MCB or water for injection – the particles will aggregate when stored in PBS.
• MNPs for conjugation can be synthesized in water; the same conjugation efficiency is observed in PBS and water but lower conjugation efficiency occurs when synthesized in MCB. Particle should be made in water, as they will still aggregate in PBS.
• MNP conjugation to a targeting ligand can be achieved with sulfo-NHS and EDC. Sulfo-NHS increases the conjugation efficiency, but also leads to aggregation and increased particle size, so conjugation with EDC alone is recommended.

2.5 Acknowledgments

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CHAPTER 3: MAGNETIC RESONANCE IMAGING OF MULTIFUNCTIONAL PLURONIC STABILIZED IRON-OXIDE NANOPARTICLES IN TUMOR-BEARING MICE

3.1 Introduction

Magnetic resonance imaging (MRI) provides excellent differential soft-tissue contrast in order to discriminate between healthy tissue and abnormalities such as tumors. Contrast agents further improve tissue resolution obtained with MRI by influencing the properties of the adjacent tissue. Several different types of contrast agents exist: paramagnetic gadolinium magnets brighten the tissue by increasing the longitudinal relaxivity, whereas superparamagnetic nanoparticles darken the tissue by altering the transverse relaxivity.\textsuperscript{66} In oncology, MNPs are being used to aid in disease staging, treatment planning, and assessing tumor response to therapy.\textsuperscript{67-68} In particular, MNPs are increasingly being used for nodal staging because of their uptake by macrophages and migration to the lymph nodes.\textsuperscript{68} Additionally, the EPR effect can be utilized to increase MNP uptake in the tumor for MRI; however, MNPs for this purpose must be appropriately designed to avoid their uptake by macrophages, resulting in rapid clearance from the systemic circulation.\textsuperscript{69}

To address the problems of short half-life and rapid clearance by the RES, MNPs are modified with various surface coatings, such as dextran, starch, citrate, or synthetic polymers.\textsuperscript{66} The surface coating affects the hydrodynamic diameter of the MNPs and their interaction with proteins circulating in the bloodstream, both of which lead to
opsonization, macrophage uptake, and clearance by the RES. By altering the surface coating, it is possible to limit MNP interaction with the surrounding environment after intravenous administration and prevent uptake by the RES, influencing the systemic circulation time and eventual localization efficiency of the MNPs to the tumor.

3.2 Materials and Methods

3.2.1 Protein Binding Studies and Magnetic Nanoparticle Uptake by Macrophage Cells

Each MNP formulation was studied for binding to BSA using fluorescence spectroscopy. The BSA solution (330 mg/mL, in mannitol–citrate buffer) was titrated with 1 mg/mL aliquots of MNP suspension such that the total protein concentration remained less than 2%. The fluorescence emission spectra of BSA at different MNP concentrations was recorded at the excitation wavelength, $\lambda_{\text{ex}} = 295$ nm and over emission wavelengths of 300–500 nm using a fluorescence spectrophotometer (PerkinElmer Life and Analytical Sciences, Inc, Waltham, MA). The intrinsic fluorescence of tryptophan residues in BSA is quenched on binding to specific sites of MNPs. This characteristic provides a method to determine binding constant ($K_b$) and number of binding sites ($n$), according to the methods of Lehrer and Fasman and Chipman et al.

The mouse leukemic monocyte macrophage cell line RAW 264.7 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 mg/mL penicillin G, and 100 mg/mL streptomycin at 37 °C in a humidified and 5% CO$_2$ atmosphere. Macrophage cells were seeded in 6-well plates (200,000 cells per well) and
allowed to reach ~80% confluence. Medium was replaced with 0.1 mg/mL MNP suspensions in media (2 ml/well) and incubated for 4 h at 37 °C. After 4 h, macrophage cells were washed gently two times with PBS, the cells were scraped and centrifuged (1000 rpm, 10 min), and the resulting pellet was used to determine MNP uptake. With concentrated hydrochloric acid added, the cell pellet was incubated at 37 °C for 30 min, and the resulting solution was diluted for iron estimation.62

Calibration curves for each formulation were prepared by plotting a known concentration of MNPs and measuring the absorbance at 511 nm. A portion of cell suspension was lysed using 1% Triton X 100 (Sigma), and protein estimation performed using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

3.2.2 Preparation of 3-D Tumor Model and Inoculation

MCF7 cells grown on a microparticle scaffold were used to generate a 3-D model for implantation in athymic nude mice.74 This 3-D structure for tumor induction forms tumors of consistent size more frequently than cells injected in Matrigel alone.

3.2.2.1 Preparation of the Microparticle Scaffold

The following solutions were prepared: A 2.5% chitosan solution was prepared by adding 62.5 mg chitosan to 2.5 mL of 10% v/v lactic acid. A 10% PVA w/v solution was prepared by adding 2 g poly(vinyl alcohol) (average MW 30,000-70,000) to 20 mL of cold water (0-4 °C) with stirring. After addition of the PVA, the solution was kept at room temperature and the suspension was stirred overnight on a stir plate. A 1% w/v
solution of PVA was prepared by the same method by dissolving 2.5 g PVA in 250 mL of cold water with magnetic stirring at room temperature.

After overnight stirring, any remaining undissolved PVA was removed by centrifuging the 10% PVA solution at 1,000 rpm for 10 min and collecting the supernatant. For preparation of a 2 mL inner aqueous phase (W1), 1 mL of the 10% PVA solution was placed in a glass vial, and 50 mg bovine serum albumin (BSA) and 200 mg sucrose were added to the PVA and vortexed until dissolved. Next, 1 mL of the 2.5% chitosan solution was added and the solution vortexed. The oil phase (O) was prepared by dissolving 200 mg poly(lactic acid) (PLA, MW 0.18 dL/g) in 4 mL of dichloromethane with vortexing. For the second aqueous phase (W2), 25 g of sucrose was added to the 1% PVA solution for a final 10% w/v sucrose mixture.

The primary emulsion (W1/O) was prepared by combining 800 µL of the W1 solution to the polymer solution (O), and an emulsion was created with homogenizing with the lowest speed of a tissue tearor (~5000 rpm) for 1-2 min (Biospec Products, Inc, Bartlesville, OK, Model 985370). 2.5 mL of this W1/O primary emulsion was immediately added drop wise to the W2 solution while stirring on a magnetic stir plate to form a multiple emulsion (W1/O/W2). The solution was stirred for 18 h in the fume hood to evaporate the organic solvent.

After stirring, the W1/O/W2 emulsion was separated into 50 mL falcon tubes and centrifuged at 1000 rpm for 15 min at 10 °C. The supernatant was removed and the
microparticles were combined in a final volume of 10 mL with gentle vortexing, and centrifuged and washed with 10 mL water two more times. After the final wash, the microparticles were resuspended in 10 mL water and separated into four pre-weighed falcon tubes, frozen at -80 °C and lyophilized.

3.2.2.2 Seeding Cells on a Microparticle Scaffold

MCF7 cells were grown in DMEM supplemented with 10% FBS and 1% pen-strep. Lyophilized microparticles (~20 mg) were resuspended in a mixture of 50% FBS and 50% DMEM at a concentration of 2 mg microparticles /mL, vortexed, and incubated at 37 °C, 5% CO₂ for 3 h. The microparticles were centrifuged for 15 min at 1000 rpm, 20 °C and the supernatant carefully removed. The microparticles were resuspended in DMEM (2 mg microparticles/mL) and transferred petri dishes placed in an inclined position (1 mL microparticle suspension in each petri dish, BD Falcon standard disposable Petri dish). The microparticles were allowed to settle for several minutes, the supernatant was carefully removed, and a 500 µL suspension of MCF7 cells in supplemented media (1×10⁶ cells/mL) was added to each dish. The petri dishes were placed in the incubator in an inclined position to for 3 h to allow the cells to attach to the microparticles. After 3 h, 4.5 mL of supplemented media was added to each dish, and returned to the incubator in the inclined position. After 48 the media was changed, and the petri dishes were returned to the incubator in a normal flat position. The media was changed every 48 h following; the petri dishes were first placed in an inclined position, and any cells attached to the petri dish were agitated to detach. After settling, the old media was removed and 5 mL fresh media placed in each dish.
After 7 days, two petri dishes were used to determine the cell count on the microparticle scaffold. The cells were washed twice with 5 mL PBS, then 2 mL of Trypsin, and detached for up to 45 min with Trypsin. When the cells had sufficiently detached, 8 mL of media was added to the mixture and the cells counted. For inoculation, a suspension of microparticles was prepared at a concentration of 500,000 cells per 100 µL of PBS. The cells were transferred to ice and Matrigel (BD Matrigel, Growth Factor Reduced, Fisher Scientific) an equal volume of Matrigel was added to the suspension such that the final volume was 500,000 cells on microparticles for each 100 µL PBS and 100 µL Matrigel.

3.2.2.3 Tumor Inoculation in Athymic Mice

The Cleveland Clinic Institutional Animal Care and Use Committee approved all animal procedures. Female athymic nude mice 20-30 g, nu/nu, Charles River, Wilmington, MA) were anesthetized by an intraperitoneal injection of 100-150 mg/kg body weight of ketamine and 10 mg/kg xylazine. Cells were grown on scaffold for 6 days. The scaffold, with 500,000 cells, was suspended in 100 µL of D-PBS and combined with 100 µL of Matrigel (BD Biosciences, Bedford, MA) and injected in the uppermost left mammary complex of each mouse. A 17-β-estradiol pellet (1.5 mg, 90-day release; Innovative Research of America, Sarasota, FL) was implanted subcutaneously in the right flank of each mouse to promote tumor growth. Tumor growth was regularly measured with calipers, and tumor volume (mm$^3$) was calculated according to the following formula:

$$Tumor Volume = \frac{length \times width^2}{2}$$
3.2.3 Magnetic Resonance Imaging

Magnetic resonance imaging studies were done when the mean tumor volume reached ~250 mm$^3$. During imaging, mice were anesthetized with isoflurane and their temperature and respiration were monitored for the duration of the experiment. A phantom (syringe filled with water) was placed alongside the mouse during imaging and used for image normalization. The tail vein was catheterized using a 26-gauge needle connected to PE20 tubing that had been flushed with heparinized saline. A suspension of MNPs or Feridex IV (40 mg Fe/kg, w100 mL) in mannitol–citrate isotonic solution was injected through the tail vein over 40 sec. Mannitol–citrate buffer (100 mL) was used as a control. Dynamic scanning of mice was performed using a 9.4T Bruker Biospec MRI scanner (Bruker Biospin, Billerica, MA) to observe the changes in the signal intensity in the tumor following administration of the contrast agents. A dynamic FLASH acquisition (TR/TE = 221.4/2.1 ms, field of view = 3.0 × 3.0 cm, matrix = 256 × 256, alpha = 20º, slice thickness = 1 mm) was used to acquire images of the tumor for each animal. After 3–4 pre-injection FLASH scans, the mouse was removed from the MRI scanner and injected with either saline, Feridex IV, or F127-or T908-modified MNPs. This step was performed so that the MNPs could be injected without aggregating in the PE20 tubing due to the magnetic field of the MRI scanner. The animal was then placed back at the same axial distance within the scanner, and the tumor imaged at 1, 2.5, 3, and 4 h post-injection of the contrast agent.

Preliminary tumor studies were analyzed for changes in signal intensity with Amira software (Mercury Computer Systems, Chelmsford, MA). Subsequent studies
were analyzed with MATLAB (The MathWorks, Natick, MA), as stated in the figure legends. The images acquired were normalized with respect to the noise and the phantom according to the following equation: Normalized Image=(Original Image–Noise)/(Phantom–Noise). Two regions of interest (ROIs) were manually drawn for each tumor slice, one surrounding the whole tumor, and the other just inside the tumor periphery (Figure 3.1, page 52). The signal intensities within the whole tumor and within the hollow tumor periphery were determined using the same ROI at each time point. This process was repeated for each tumor slice (7–8 slices per tumor), and an area-weighted average was calculated over the whole tumor volume.

3.2.4 Histological Analysis

Tumors were collected 24 h after injection of the contrast agent. Mice were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg), then perfused with PBS and 4% paraformaldehyde by intracardiac injection to clear the blood from the vessels. The tumor and surrounding tissue were excised and stored in 10% paraformaldehyde. Paraffin-embedded histological slices (~4 mm thick) were stained with hematoxylin–eosin (H&E). Prussian blue staining was used to identify the iron in the histological sections of the tumor. The samples were deparaffinized and rehydrated, then immersed in a solution containing 5% w/v of potassium ferrocyanide and 10% hydrochloric acid (v/v) in water for 3 h.\textsuperscript{75} Slides were rinsed with water and images obtained on a Leica DMR upright microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Retiga EXi Cooled CCD Camera (QImaging, Burnaby,
Figure 3.1 Whole tumor and tumor periphery regions of interest.

The signal intensity was calculated for the whole tumor, the region enclosed by the outermost green polygon, and for the tumor periphery, the region between the two polygons.
British Columbia, Canada) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

3.3 Results

The binding constants ($K_b$) varied for each MNP formulation (Table 3.1, page 54). Feridex IV and F127-modified MNPs bound strongly to BSA, while T908-modified MNPs bound weakly. The number of binding sites on the MNPs was similar among the formulations (range, 1.15–1.72). Feridex IV and T908-modified MNPs had the highest number of binding sites; pluronic F127-, L64-, and F68-modified MNPs had fewer binding sites. To determine which MNPs might avoid premature macrophage uptake and clearance in vivo, we analyzed MNP uptake in RAW 264.7 cells in vitro. Feridex IV uptake was 0.05 $\mu$g Fe/$\mu$g cell protein, about one fourth as much as any of our MNP formulations. By comparison, macrophage uptake for most pluronic- or tetronic-modified MNPs was about 0.20 $\mu$g Fe/$\mu$g cell protein. The uptake by macrophages of pluronic F68-modified MNPs was two fold that of the other formulations, with a measured macrophage uptake of 0.48 $\mu$g Fe/$\mu$g cell protein.
Table 3.1 Effect of Pluronic and Tetronic Coatings on Magnetic Nanoparticle Characteristics *In Vitro*

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<th>Co-polymer</th>
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<td>Binding Constant ($K_b$)</td>
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<tr>
<td>Pluronic F108</td>
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<td>Pluronic L64</td>
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<tr>
<td>Tetronic 904</td>
<td>0.075</td>
</tr>
<tr>
<td>Tetronic 908</td>
<td>0.036</td>
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</table>

* Macrophage uptake ($\mu$g Fe/ $\mu$g cell protein), 0.1 mg/mL particles incubated with the mouse leukemic monocyte macrophage cell line RAW 264.7.
3.3.1 Magnetic Resonance Imaging

Based on the *in vitro* characteristics of the MNPs, particularly the size and hydrophilic content, we selected F127- and T908-modified MNPs to image in a mouse xenograft tumor model. An initial study confirmed decreased signal intensity and enhanced tumor contrast for F127-modified MNPs immediately after intravenous injection (Figure 3.2, page 56). The darkened tissue indicating MNP uptake is observed in both the tumor (denoted by arrow) and the liver. Re-injection of the contrast agent at 60 min further decreased the signal intensity. This preliminary study confirmed that the MNPs enhanced the contrast in the tumor tissue. In subsequent experiments, a phantom was placed next to the mouse, the image normalized, and the change in contrast quantified.

The contrast observed within the tumor of mice injected with Feridex IV, T908- or F127-modified MNPs varies at each axial slice due to the heterogeneity of the tumor vasculature (Figure 3.3, page 57). The decrease in signal intensity is more apparent for each contrast agent in the outer slices of each tumor (Figure 3.3, S 02–04 and S 07–08). The contrast in the vascular tumor periphery decreases sharply at 1 h for Feridex IV and T908-modified MNPs and returns to baseline over the next 3 h (Figure 3.3 and Figure 3.4, pages 57 and 58). The reduced signal intensity of the whole tumor in each slice and over the entire volume was not as pronounced for Feridex IV and T908-modified MNPs as with F127-modified MNPs. F127-modified MNPs display enhanced contrast in both the vascular tumor periphery and in whole tumor at 1 h, which persists over the 4 h imaging period (Figure 3.3, S All). In addition, F127-modified MNPs were the only
Figure 3.2 T2-weighted image of tumor-bearing mouse injected with pluronic F127-modified MNPs.

Enhanced contrast in the tumor (denoted by arrow) is apparent 4 min after the initial injection and is more pronounced at 68 min after a second injection of the MNPs. Images were analyzed for signal intensity in the tumor with Amira software (Visage Imaging, Inc., San Diego, CA).
Figure 3.3 MNPs taken up within the tumor tissue enhance magnetic resonance imaging contrast.

Contrast enhancement within the whole tumor and vascular tumor periphery for mice injected with saline, Feridex IV, F127-modified MNPs, or T908-modified MNPs. A single ROI was drawn around the tumor at each axial slice (S 02-S 09) for the pre-injection image (0 h) and the signal intensity quantified. The same ROI was used to calculate the signal intensity at 1, 2.5, 3, and 4 h. The signal intensity from a second ROI drawn just inside the first was subtracted from the first to determine the signal intensity within the tumor periphery. An area-weighted average of the slices gave the signal intensity for the whole tumor (S All). X-axis: each tick mark represents 1 h, beginning at 0 h and ending at 4 h post-injection of saline or the iron-oxide contrast agent. Images analyzed in MATLAB (The MathWorks, Natick, MA). Data represented from at least two repeats.
Figure 3.4 Magnetic resonance imaging sections after iron-oxide nanoparticle injection.

Transverse relaxivity of the MNPs enhance the contrast in mouse xenograft tumors (arrow indicates tumor). The initial enhanced contrast in the tumors of mice injected with Feridex IV or T908-modified MNPs fades over 4 h, while the enhanced contrast in the tumor of a mouse injected with F127-modified MNPs remains throughout the 4 h of imaging. Red indicates high signal intensity, blue lower signal intensity. Images analyzed in MATLAB.
particles in which there was a greater decrease in signal intensity in some slices in the whole tumor than in the tumor periphery (Figure 3.3, S 03, S 05, and S 06). Mice injected with saline did not show any change in signal intensity over the 4 h imaging period in either tumor periphery or whole tumor.

3.3.2 Histology

Each tumor showed a viable periphery and a necrotic core visible in the H&E-stained sections (Figure 3.5, page 60). Prussian blue-stained sections indicate iron in the tumor tissue 24 h after injection of the MNPs. The Prussian blue staining appeared only in the tumors of animals that received MNPs or Feridex IV, but not in those of the saline control mice.

3.4 Discussion

The surface properties of MNPs critically influence the interactions of MNPs with proteins, cells, and the localization of MNPs to tumors.71 To prevent early uptake by macrophages and too rapid clearance of MNPs, the steric stabilization must be optimized so that the polymer used for surface modification is adequately anchored to the surface, the surface is fully coated, and the coating is sufficiently thick.76 In this study, we analyzed different surface coatings of our MNPs to limit their clearance by the RES and enhance contrast in xenograft breast tumors. Our study demonstrated that F127-modified MNPs provide whole tumor contrast over an extended time frame of at least 4 h.
Figure 3.5 Tumor histology after injection with iron-oxide contrast agents.

Blue-violet staining of the viable periphery and red-pink staining of the necrotic core is evident in each H&E-stained tumor section. Iron from the MNPs or Feridex IV stained blue in the tumor periphery 24 h after MNP injection. The scale bars are 25 µm in all H&E-stained images, and 10 µm in all Prussian Blue-stained images.
Our MNP is composed of an iron-oxide core coated with OA. The OA-magnetite particles are hydrophobic and not dispersible in water. However, this formulation easily allows a hydrophobic/hydrophilic block copolymer to anchor onto the OA-magnetite core for aqueous dispersion (Figure 2.1, page 21). We modified the surface coating of our MNP formulation with various pluronic and tetronic block copolymers to identify a formulation with minimal protein interaction, extended circulation time, and enhanced uptake into the tumor. We compared our formulations to Feridex IV, a commercial contrast agent.

Uptake of MNPs by macrophages *in vitro* did not differ significantly among the various pluronic and tetronic copolymers used to coat our MNPs, though they all showed somewhat greater uptake than Feridex IV. Limited PEO and PPO domains significantly influence aggregation, and the large particle size typically leads to increased serum protein adsorption and uptake by macrophages. The PPO chain strengthens the copolymer interaction with the OA-coated MNP, allowing more copolymer to bind and stay bound during MNP synthesis. If the PPO anchoring is inadequate, more of the hydrophobic OA-magnetite core is exposed and the MNPs aggregate.

We selected one pluronic- and one tetronic-coated MNP formulation for *in vivo* MR imaging of the MCF-7 breast tumor in mice. Based on *in vitro* MNP size and copolymer anchoring, F68, L64, and T904 polymers were not considered suitable MNP surface coatings for *in vivo* studies because the RES would too quickly clear the particles. Particles modified with F127 had a very high pluronic content and low number of binding
sites compared to F108-modified MNPs, therefore, we selected F127-modified MNPs as the pluronic coated MNP for MRI studies. Tetronic T908-modified MNPs were selected for MRI over T904-modified MNPs because they did not aggregate and had a smaller $K_b$, with low macrophage uptake.

In the present study, we took advantage of the defective tumor vasculature for particle accumulation in the tumor, the EPR effect.\textsuperscript{78} Other experiments have shown that the EPR effect is an effective means to deliver iron-oxide particles to tumors for MRI, generally with transient accumulation in the first hour followed by clearance of the particles in the next 2 h. Iron-oxide particles modified with poly(ethylene glycol)—poly(aspartic acid) block copolymers or with copolymers thermally cross-linked to the surface of the MNP enhance tumor contrast for 1–3 h or 3.5 h after injection, respectively.\textsuperscript{79-80} MRI studies with pegylated gadolinium nanoparticles enhance the contrast in the tumor periphery in the first 40 min, but the contrast fades to that of pre-injection levels 2–6 h post-injection.\textsuperscript{81} Feridex IV and our T908-modifed MNPs paralleled these findings; strong contrast in the tumor periphery 1 h post-injection returned towards baseline by 2.5–3 h postinjection. F127-modified MNPs enhanced contrast in the tumor more than T908-modified MNPs and Feridex IV, and the contrast remained for the duration of the imaging.

\textit{In vivo}, the depth of PPO anchoring onto OA may prevent the shear forces the MNPs are exposed to from breaking the physical interaction between the PPO and OA, which may be why the contrast remained for F127-modifed MNPs and faded for T908-
modified MNPs. In addition, F127-modified MNPs have a much greater wt% of pluronic coating than any of the other MNPs (Table 2.2, page 33) because of the strong PPO anchoring. This increase in the overall wt% of the coating increases the hydrophilic content, which is known to contribute significantly to the extended circulation time by preventing protein adsorption (opsonization) and phagocytosis. This characteristic could explain the transient contrast effect of T908-modified MNPs over F127-modified MNPs because of the difference in their block copolymer content (7.5 wt% for T908-modified MNPs vs. 14.5 wt% for F127-modified MNPs). In addition to extending the circulation time and preventing protein adsorption, phagocytosis, and RES clearance, the stability of the F127-modified MNPs in circulation may also allow the particles to diffuse through the tumor vasculature so that F127-modified MNPs act as a whole tumor contrast agent. This is a significant improvement compared to the T908-modified MNPs and Feridex IV, which do not show significant whole tumor contrast.

Despite smaller mean size and lower macrophage uptake of Feridex IV compared to our formulation, our previous study showed longer systemic circulation half-life of F127-modified MNPs than Feridex IV in mice (31.2 min vs. 6.4 min). The circulation half-life in the study was determined from the changes in MRI signal intensity in both carotid arteries of mice following a tail vein injection of 7 mg Fe/kg. The longer circulation half-life of F127-modified MNPs than Feridex IV also explains the better tumor contrast seen in this study with F127-modified MNPs.

The rapid clearance of Feridex IV could be due to its heterogeneous particle size.
distribution compared to F127-modified MNPs (polydispersity index, 0.3 vs. 0.1, Table 2.2, page 33), thus rapidly removing Feridex IV from circulation. Alternatively, the pluronic F127 coating surrounding the iron-oxide core could provide greater particle dispersion stability in vivo than the dextran coating used for stabilizing iron-oxide particles in Feridex IV. McCarthy et al. have reported that one of the main drawbacks is that the dextran coating is in equilibrium with the surrounding medium, as it is not strongly associated with the iron-oxide core. Additionally, MNPs are subjected to high shear forces in vivo, which are not replicated under in vitro conditions. Weak binding of the coating agent to the magnetic core could become a critical factor in the destabilization of the formulation when subjected to high shear force in vivo. Further studies on the stability of the coating and that of particles under shear force replicating in vivo conditions could perhaps provide better insight into the rapid in vivo clearance of Feridex IV, which, despite its more favorable characteristics of smaller mean particle size and lower macrophage uptake in vitro, did not compare as well to our formulation of MNPs in vivo.

We have previously demonstrated the biocompatibility of F127-modified MNPs in rats; the MNPs caused transient changes in the liver enzyme levels and oxidative stress. This finding is important because one of the limitations of the gadolinium-based contrast agents is their serious renal toxicity.

The prolonged presence of MNPs in tumors is an advantage not only for MRI, but also to deliver anticancer drugs, since our formulation has the unique advantage of
entrapping high doses of hydrophobic anticancer drugs in the OA layer for sustained release while retaining favorable MRI properties.\textsuperscript{39, 60} We have previously shown loading of paclitaxel and doxorubicin (base), and also the combination of the two in our MNPs with drug loading of 8.2–9.5 % w/w and sustained release of the loaded drugs.\textsuperscript{60}

In addition to drug delivery and MRI, MNPs can also be actively targeted to the tumor by conjugating antibodies to the MNPs for receptors over expressed on the cell surface, or by applying an external magnetic field to localize the MNPs to an area of interest.\textsuperscript{86–87} The conjugation to a targeting ligand can enhance the efficiency and specificity of MNPs. An alternating magnetic field can be applied to tumor cells containing MNPs to heat and selectively kill cancer cells, which are more sensitive to increased temperature than healthy cells.\textsuperscript{88} Targeted nanocarriers with simultaneous imaging and drug delivery capabilities are of clinical importance as it could allow detection of pathologies as well as delivery of therapeutics. Our formulation of MNPs can thus be developed as a theranostic agent with combined properties of drug delivery and imaging.

\textbf{3.5 Conclusions}

F127-modified MNPs demonstrated sustained and enhanced contrast throughout the tumor mass, whereas contrast was transient and confined to the tumor periphery with Feridex IV. Since our MNPs can also be loaded with anticancer agents without influencing the MRI properties, these MNPs can be developed as an effective theranostic agent that could potentially be used for tumor detection, drug delivery, and evaluation of
response to therapy.

3.6 Acknowledgments

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CHAPTER 4: OPTICAL IMAGING AND MAGNETIC FIELD TARGETING OF MAGNETIC NANOPARTICLES IN TUMORS

4.1 Introduction

Nanoparticles are a promising theranostic agent with applications for tumor imaging and targeted cancer drug delivery. New designs in nanoparticle formulations take advantage of synergism of multiple imaging modalities to improve cancer diagnosis and treatment and to monitor response to therapy. Two such complementary techniques are magnetic resonance imaging (MRI) and optical imaging. Magnetic resonance imaging provides excellent deep tissue contrast and spatial resolution; however, it is not a quantitative technique, and it is quite costly. Optical imaging is inexpensive, sensitive, and provides excellent spatial and temporal resolution, but penetration is limited to a few millimeters below the tissue. By designing nanoparticle formulations with dual imaging applications, the advantages of MRI as a diagnostic tool can be combined with optical imaging to quantitatively track and determine the biodistribution of nanoparticles in vivo.

Formulations of MNPs that combine MRI and optical imaging encapsulate iron-oxide and fluorophores in emulsions or polymeric nanocapsules, or conjugate fluorophores to the surface of the nanoparticles. These techniques produce constructs that can be successfully imaged with MRI and optical imaging; however, some investigators report challenges with these techniques. Iron-oxide blocks the fluorescent signal in encapsulated formulations, and chemical conjugates, particularly amide bonds, are susceptible to cleavage in vivo. Chemical conjugation can be technically
challenging and may alter the charge and biodistribution profile of nanoparticles.\textsuperscript{63, 96} Multiple agents may compete for the same surface binding sites, altering the efficacy of one or both agents.\textsuperscript{97} In addition, the surface-conjugated dye may be cleaved quickly, hindering the long-term \textit{in vivo} biodistribution study of MNPs. Thus, it is beneficial when designing a particle for multifunctional applications to encapsulate agents and limit the number of targeting, therapeutic, and imaging ligands conjugated to the surface.

The ultimate goal of our MNP design is to increase the delivery of MNPs, and therefore drug, to tumors, as well as aid in diagnosis and evaluation of tumor response with complementary imaging techniques. Incorporating fluorophores within our MNP formulation will also allow us to determine how changes in the formulation and targeting mechanisms alter the biodistribution and accumulation of the MNPs in the tumor over time. The goals of the present study are to (a) select near-infrared (NIR) hydrophobic dyes with strong fluorescence intensity and low toxicity that can be loaded into our MNPs for optical imaging, (b) determine the \textit{in vivo} biodistribution of MNPs in tumor-bearing mice by optical imaging, and (c) optically compare the passive accumulation of MNPs within the tumor to MNPs actively targeted to the tumor by an externally applied magnetic field (MF).

4.2 Materials and Methods

4.2.1 Fluorescence Intensity and Photobleaching

The fluorescence intensity of the NIR dyes was measured by dropping 3 µL of dye (2.5-50.0 µg/mL in ethanol) onto filter paper and imaging with the Maestro EX NIR
filter sets; the intensity (Scaled Counts/s) was determined from a circular ROI for each sample. The peak emission for each NIR dye was measured on the Maestro EX (Cambridge Research and Instrumentation, Woburn, MA). To determine if the iron oxide quenched the fluorescence of dyes \textit{in vivo}, mice were anesthetized with isoflurane and subcutaneously injected with 20 µL of dye-MNPs (1.0 mg MNP/mL; 2.5-50 µg dye/mL). ROIs surrounding the injection site were used to calculate the signal intensity (Scaled Counts/s).

\subsection*{4.2.2 Dye Toxicity}

MCF7 breast cancer cells were treated with 2.0\% w/w dye-MNPs at various concentrations to determine dye toxicity. MCF7 cells (100 µl) were seeded in 96-well plates (3000 cells/well) in DMEM (supplemented with 10\% FBS and 1\% penicillin streptomycin) and allowed to attach for 1 day. Media was removed and cells were treated with 2.0\% w/w dye-MNPs (5-10,000 ng/mL dye, 0.25-50 µg/mL MNPs). The dye-MNPs were removed after 1 day, and cells were given fresh supplemented media. The media was changed every 2 days thereafter, and the cells were not given any additional treatment. Five days after treatment, the media was changed, and cell viability was determined using an MTS assay (CellTiter 96 Aqueous, Promega, Madison, WI). A curve was mathematically fit to the data to determine the dye concentration that resulted in 50\% inhibition of cell growth (IC$_{50}$) using the following equation:

$$y = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_o}\right)^p} + A_2$$

where $y = \%$ Growth at dye concentration $x$, $A_1 = \text{maximum } \%$ Growth, $A_2 = \text{minimum } \%$ Growth, $x_o = \text{inflection point of the curve}$, and $p = \text{largest absolute value of the slope of}$
the curve.

4.2.3 Animal Experiments

Mice bearing MCF7 breast tumors (tumor induction procedure described in section 3.2.2.3 on page 49) were treated when tumors reached ~300 mm$^3$. Mice were kept on the Teklad Global 18% protein rodent diet (2018S), an alfalfa-free, wheat-based diet (Harlan Laboratories, Indianapolis, IN), to reduce autofluorescence.

4.2.4 Biodistribution of NIR Dye-MNPs

The distribution of dye-MNPs in a mouse with an MCF7 xenograft breast tumor was determined with the Maestro EX. A mouse anesthetized with isoflurane was injected intravenously with 100 µL of 5491 dye-MNPs (4.9 mg MNPs/mL, 0.25 mg dye/mL) and imaged daily until the dye-MNPs were no longer detected. The Maestro Blue filter set was used to image the mouse autofluorescence and the NIR filter was used to detect the 5491 dye-MNPs.

4.2.5 Influence of a Magnetic Field on the Localization of NIR Dye-MNPs in Tumor-Bearing Mice

The effect of an externally applied MF on the localization of NIR dye-MNPs in an MCF7 xenograft breast tumor was determined by placing a magnet (Neodymium Iron Boron, 1.25 × 1.25 × 0.3 cm, Gauss: 12200, Edmund Scientific, Tonawanda, NY) over the surface of a tumor with Steri-Strip tape (3M Health Care, St. Paul, MN) while mice were anesthetized with isoflurane. Control mice, also anesthetized with isoflurane, did
not have a magnet placed over the tumor. While anesthetized, mice were injected with 100 µL of 0.25 % w/w 6825 dye-MNPs in mannitol citrate buffer (86.8 mg/kg MNPs, 0.22 mg/kg dye). After 1 h, the magnet was removed from the mice. Mice were imaged with the Maestro EX NIR filter set 24 h post injection, injected with a lethal dose of sodium pentobarbital, and perfused via intracardiac injection of saline to remove circulating MNPs. The tumors were collected and immediately imaged with the Maestro EX NIR filter set. Tumors were weighed and homogenized in sterile Milli-Q water at 0.1 g/mL. The homogenate was serially diluted to a final volume of 100 µL in each well in a white 96-well plate, and the fluorescence measured with the Maestro EX NIR filter set. Dye-MNPs (0.25% w/w 6825 dye-MNPs, 0-100 ng dye) were suspended in tumor homogenates of an untreated animal for a standard plot. The average of the diluted samples in the linear region of the standard plot was compared with the unknown samples to determine the amount of dye present in each tumor. Data were normalized based on the position of each well under fluorescent light.

4.2.6 Statistical Analysis

Statistical analyses were performed using Student’s t test. The differences were considered significant for p values of ≤ .05.

4.3 Results and Discussion

In this study, we tested multiple dyes in the NIR window to optimize our MNP formulation for biodistribution analysis. We used a mouse xenograft breast tumor model to test passive MNP accumulation versus active targeting with a MF.
For *in vivo* experiments, mice were anesthetized and continuously exposed to NIR light over 20 min to determine if photobleaching would be an issue while imaging over several days. We selected this time frame because each daily image would result in NIR light exposure for less than two minutes over several days for biodistribution studies. We saw a <1% change in signal intensity over the body of the mouse over the 20 min of imaging. Because body tissues scatter and absorb the light, the tissue appears to act as a protective barrier, actually limiting photobleaching of the dyes *in vivo*. We concluded that images taken daily to track the long-term biodistribution of MNPs would be accurate and quantifiable, and we would not need to correct for photobleaching. To evaluate the potential toxicity of the dyes, the IC$_{50}$ for each of the dye-MNP formulations was determined *in vitro* in an MCF7 cell line (*Figure 4.1*, page 73). At the concentrations tested, MNPs did not inhibit cell growth, whereas MNPs loaded with dye 5700 were the most toxic with the lowest IC$_{50}$, followed by MNPs loaded with dye 2826, 6825, 5491, and 5177.

We imaged each dye *in vitro* and dye-MNPs *in vivo* at varying concentrations to evaluate which dyes were more intensely fluorescent (*Figure 4.2*, pg. 74). The baseline intensity of dye 5700 was much higher than that of the other dyes tested. Small increases in the concentrations of dye 6825 provided the greatest increase in the fluorescence signal detected (*Figure 4.2 A and C*, pg. 74). The *in vivo* fluorescence signal intensity of dye-MNPs injected subcutaneously generally paralleled the trends of the *in vitro* fluorescence signal, with a decrease in the signal intensity due to scattering and absorbance of the fluorescence within the tissues (*Figure 4.2 B and D*, pg. 74).
Figure 4.1 NIR dye toxicity \textit{in vitro}.

The MCF7 breast cancer cells were treated with MNPs containing 2% w/w NIR dye, for 24 h. The cell viability was measured five days later.
Figure 4.2 Fluorescence of NIR dyes and NIR dye MNPs.

Fluorescence of NIR dyes in vitro (A) and dye-loaded MNPs in vivo (B). A small amount (3 µl) of each hydrophobic dye in ethanol was dropped onto filter paper and the fluorescence intensity measured with manually drawn ROIs. Magnetic nanoparticles containing 0.25-5.0% w/w dye were suspended in mannitol citrate buffer (1 mg MNPs/mL) and 20 µL was subcutaneously injected and immediately imaged. Representative images are shown for dye 2826 in ethanol (C), and a mouse subcutaneously injected with MNPs loaded with dye 5700 at 0.25, 0.5, and 1.0% w/w dye in 1 mg/mL MNPs (D).
Based on the toxicity and in vivo fluorescence intensity studies, we concluded that dyes 5700, 6825, and 5491 were more suitable than dyes 2826 and 5177 for tracking the in vivo biodistribution of the MNPs. We did not observe any toxic side effects in the animals injected with dye-loaded MNPs. These observations, in combination with previous studies evaluating the biocompatibility of MNPs in rats, suggest that both plain and dye-loaded MNPs do not cause deleterious effects in rats or mice at the MNP concentrations tested.

An important application relative to our design is the ability to visualize multiple fluorescent species simultaneously, as the dyes are spectrally distinct. Dyes 6825 and 5700 can be easily unmixed from dye 5491, but their spectra overlap enough such that they cannot be unmixed from each other. Because the different fluorophores can easily be loaded into MNPs, conjugating the particles to different receptors could aid in tumor identification in vivo, and in distinguishing between subsequent doses of nanoparticles loaded with dye and therapeutic agents.

We next evaluated the in vivo biodistribution of our MNPs in a mouse model with xenograft breast tumors. MNPs containing dye 5491 (5.0% w/w) were intravenously injected via tail vein and imaged daily with the Maestro blue and NIR filter sets. One day after injection, the dye-MNPs were easily visible in the subcutaneous MCF7 breast tumor (Figure 4.3 A pg. 76). Regions of interest (ROIs) drawn over the tumor, bladder, liver, and heart were used to quantify the change in signal intensity each day until no signal was seen (Figure 4.3 B and C, pg. 76). The dye-MNPs in the tumor are more easily
Figure 4.3 Biodistribution of NIR dye MNPs.

Distribution of Pluronic F127-coated MNPs loaded with the NIR dye 5491 in an athymic nude mouse with a xenograft breast tumor. A mouse was injected with a 100-µL suspension of MNPs in mannitol citrate buffer (4.9 mg MNPs/mL, 5.0% w/w dye) and imaged each day with the Maestro EX imaging system using the blue and NIR excitation and emission filter sets (A). Autofluorescence is shown in green, MNPs loaded with dye 5491 in red. Arrow denotes tumor. Signal intensity of these MNPs in the tumor (T), bladder (B), liver (L), and heart (H) was determined from ROIs drawn over the area of each organ (B and C). Biodistribution was tested in multiple tumor bearing mice with varying dyes and dye concentrations. The same general trends of high signal intensity measured in the tumor were observed. Figure is representative data with one of the dyes.
quantified because the tumor is subcutaneous. Regions over the bladder, liver, and heart provide a baseline for background fluorescence but are not accurate representations of the amount of MNPs present in these organs because a great deal of the signal is lost due to scatter and absorbance within the tissue for these deeper organs. Fluorescence molecular tomography, or 3-D imaging machines, may provide more accurate biodistribution of MNPs to other body compartments.

The distribution of dye-MNPs follows a traditional one-compartment pharmacokinetic model with prolonged absorption and an increase in signal intensity over the first 2 days, followed by elimination of the particles and dye from the tumor region over 11 days. Generally, the tumor signal to background fluorescence ratio was low during the first 24 h, but increased dramatically after strong signal clearance by the liver and intestines. The hydrophobic dyes were dissolved in ethanol and loaded into our MNP formulation for in vivo applications. These dyes partition into OA around the iron-oxide core (Figure 1.2, page 16). We could not find a suitable non-toxic medium to dissolve dyes to observe their biodistribution without MNPs. Nanoparticles are known to localize in tumors due to the enhanced permeability and retention effect because of leaky tumor vasculature and impaired lymphatic drainage. Hence, the biodistribution and clearance kinetics of free dye and dye encapsulated in MNPs is expected to be different.

Interactions of nanoparticles with the components of the RES, and other factors – such as size, shape, surface properties (charge, hydrophilicity/hydrophobicity, etc.), targeting ligands, and vascular porosity – can influence the tumor targeting efficiency of
nanoparticles. The *in vivo* stability of nanoparticles is important to achieve prolonged systemic circulation and tumor targeting of nanoparticles. Therefore, significant effort is now focused on understanding how nanoparticles behave *in vivo*.99

The ability of our MNPs to remain in the circulation and extravasate into the tumor mass could be due to the combined effect of its hydrophilic and flexible structure. This could have prevented the MNPs opsonization and clearance by circulating monocytes. To support the above view, we did not see any change in particle size of our MNPs in the presence of protein.64 Our MNP structure is mostly flexible, except the magnetic core, which is only ~12 nm in diameter. In general, flexible structures provide better systemic circulation than rigid nanostructures of the same diameter. More than the size, perhaps it is the nanoparticle structure and its stability *in vivo* that has the greater influence on the systemic clearance of nanoparticles. For example, ultra-small (8.7 nm) citrate-coated superparamagnetic iron-oxide nanoparticles have the shortest half-life because of their highly anionic surface charge.100 Feridex IV mainly localizes in the liver, despite its small size (hydrodynamic mean diameter = 143 nm), because the dextran coating equilibrates with the surrounding medium,84 and hence it dissociates from the magnetic core, causing particles to aggregate following its intravenous injection.

To determine if we could use optical imaging as an *in vivo* technique to evaluate how targeting might affect the tumor accumulation of MNPs, we compared the passive accumulation of MNPs with MNPs actively targeted to the tumor by an externally applied MF. Mice were anesthetized and intravenously injected with MNPs and then
either did (+MF) or did not (-MF) undergo a 1 h MF treatment. At 24 h post injection, the mice were imaged for accumulation of MNPs (Figure 4.4, pg. 80). Images of tumors in vivo (Figure 4.4, A and C), tumors ex vivo isolated following perfusion of animals (Figure 4.4, B and D), and fluorescence from the tumor homogenate were on average greater in each of the mice exposed to the MF. We examined the tumors ex vivo and the fluorescence from the homogenate to confirm that the signal originated from within the tumor and because of concern that scatter and absorbance from the surrounding tissue might not provide an accurate representation of the signal present. This scatter and absorbance likely explains why the tumor fluorescence varies from in vivo to ex vivo (Figure 4.4, C vs. D). We also see a slight difference in the fold increase in tumor fluorescence with and without MF based on ex vivo imaging and the tumor homogenate (Figure 4.4, D vs. E). This may be because we detect fluorescence in the periphery of the tumor while imaging the whole organ ex vivo. Additionally, MNPs may not localize to the necrotic tumor core, and the MF provides greater strength to the periphery of the tumor. Despite these differences in the fluorescence measurements, it is clearly evident that the MF results in greater tumor localization of MNPs. The signal intensity in the tumor as a percent of the total from all other organs (heart, lungs, liver, spleen, kidneys, and brain) was 1.5% (-MF) and 3.5% (+MF) from ROIs drawn ex vivo over the whole organs. Since the animals were perfused with saline prior to isolating tumors for ex vivo imaging, the fluorescence seen is most likely due to MNPs localized in the tumor interstitial space and cells rather than in tumor vasculature. It would be interesting to confirm the spatial distribution of dye-MNPs in the tumor histologically, however, a dye
Figure 4.4 Magnetic field targeting of NIR dye MNPs in breast xenograft tumors.

Tumor localization of NIR-dye MNPs increases with 1 h MF treatment. Mice bearing MCF7 xenograft breast tumors were injected with 0.25% w/w MNPs loaded with dye 6825 and subjected (+MF) or not subjected (-MF) to the MF for 1 h. At 24 h post injection, mice were imaged in vivo (A); arrows point to tumors. Animals were euthanized and perfused with saline, and the tumors were extracted and imaged (B). The fluorescence signal was measured from an ROI drawn around the whole tumors in vivo (C), ex vivo (D), and from the homogenized tumors in a 96-well plate (E). Homogenate was normalized based on the position of the tumor in a 96-well plate. All fluorescence images were acquired with the Maestro EX in vivo imaging system with the NIR filter set. Data are shown as mean ± SEM (n = 4-5), *p = .05.
with a lower wavelength would be necessary to visualize the fluorescence by confocal microscopy.

We have shown that a short (1 h) exposure to a MF can produce a detectable difference of particles 24 h later verified with optical imaging. However, other investigators have used constant external MF exposure of 2 h, 8 h, or 10 days, then immediately euthanized the animals after removing the MF because of concerns that removing the magnet might result in reversible accumulation of magnetic particles or magnetically labeled cells.\textsuperscript{101-103} Our study demonstrates that even if the MF is removed, the shorter exposure time was sufficient to significantly increase the accumulation evidenced by strong fluorescence signal intensity 24 h post injection. Since animals were perfused before collecting the tumor, the signal seen is due to the MNPs localized into tumor tissue and not MNPs within the tumor blood vessels.

Phase 1 clinical trials with magnetically targeted drug particles were clinically effective in reducing tumor growth and particles were detected through histological methods up to 6 weeks post-injection with only 1- to 2-h MF exposure.\textsuperscript{55} This clinical trial suggests that although a significant fraction of particles are cleared by the liver and the RES during the first 48 h, the larger size of the particles, as well as firm and constant MF exposure, may be key to attracting MNPs over a long distance and to retaining particles even under conditions of an ill-perfused tumor vasculature. These results, along with our optical imaging data, suggest that significant portions of MNPs are retained in the tumor after MF removal and that MNPs in combination with drugs can be clinically
effective in reducing tumor size. This strategy could be effectively used for imaging and treatment of superficial tumors such as cancer in the breast or various sarcomas. Further, hyperthermia induced using an alternating MF to the tumor tissue can potentially enhance the efficacy of anticancer drug loaded in MNPs.\textsuperscript{104}

Optical methods are most suitable for preclinical studies to map changes in tumor vasculature or expression of receptors during tumor growth or in response to treatment.\textsuperscript{105} MNPs with dual MRI and optical imaging characteristics could be particularly useful in detection of tumors via these two modalities to delineate the tumor periphery during surgical resection.\textsuperscript{31-32} The next generation of nanoparticle-based research is directed at the consolidation of different functions into strategically engineered multifunctional nanoparticles to perform complementary roles in cancer therapy. Our MNPs have the potential to be developed as such a multifunctional theranostic agent.

4.4 Conclusions

We successfully developed MNPs with optical imaging properties and determined their dynamics of biodistribution \textit{in vivo}, their localization and retention in tumor tissue when exposed to an externally applied MF, and their eventual clearance. With the use of highly sensitive optical imaging, it may be possible to evaluate how formulation characteristics (such as the interplay of physical characteristics and targeting ligands) may increase the accumulation of MNPs in tumors and eventually enhance drug delivery. MNPs with combined drug delivery and imaging properties can potentially be developed as an effective theranostic agent.
4.5 Acknowledgment

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CHAPTER 5: MAGNETIC NANOPARTICLES SELECTIVELY INHIBIT BREAST TUMOR GROWTH

5.1 Introduction

The translation of a nanoparticle with drug delivery applications to successful cancer cell ablation presents many challenges; a particle must effectively encapsulate a drug and be non-toxic on its own. Nanoparticles for drug delivery should release the drug for selective tumor kill while minimizing damage to the surrounding healthy tissue. The efficacy of a nanoparticle for drug delivery in vitro does not necessarily translate to in vivo effectiveness. From previous studies, we knew that our MNP was compatible in healthy rats, and could enhance MCF7 tumor contrast in mice. When loaded with anti-cancer agents doxorubicin and paclitaxel, the formulation provided sustained drug release and thus cancer cell death in vitro to both breast cancer and prostate cancer cell lines. We had further found that loading with drug did not effect the MRI properties of the MNPs, suggesting that our agent could be used for both tumor identification and therapy. However, we have not yet evaluated the efficacy of the formulation in delivering chemotherapeutics in vivo.

In the present study, we investigate whether our drug loaded magnetic nanoparticle formulation could be used for the treatment of human breast tumors in an athymic nude mouse model. We found, to our surprise, that while the drug loaded nanoparticles were effective in decreasing tumor growth and increasing animal survival, so too were our plain MNPs. Thus, we came up with two hypothesis to explain this
effect. The first hypothesis was that the nanoparticles might be blocking the tumor
vasculature preventing tumor growth. The second hypothesis was that the iron from the
MNPs might be generating additional oxidative stress in the tumor environment via the
Fenton reaction, slowing the tumor growth leading to increased survival in the absence of
traditional anti-cancer therapeutics.

5.2 Materials and Methods

5.2.1 Reagents

L-012 was purchased from Wako Chemicals USA, Richmond, VA. For apoptosis
detection, the following reagents were purchased: 1% Paraformaldehyde (Methanol-free),
Phosphate Buffered Saline (PBS, 0.01M), Ethanol:Acetic Acid(2:1), ProLong Gold
antifade with DAPI (Invitrogen, Carlsbad, California), ApopTag Plus Fluorescein In Situ
Apoptosis Detection Kit (Millipore, Temecula, California), Optimal Cutting
Temperature(OCT) Compound (Sakura Finetek USA, Torrence, California).

5.2.2 In Vivo Experiment Design

Female athymic nude mice (20-30 g, nu/nu, Charles River, Wilmington, MA)
were inoculated with MCF-7 breast cancer cells and implanted with a 17-β-estradiol
pellet (1.5 mg, 90-day release) to promote tumor growth as described in section 3.2.2.3
(Page 49). For long-term survival studies, some mice survived past 90 days and were re-
implanted with an additional 17-β-estradiol pellet.
When tumor volume reached 300 – 400 mm$^3$, mice were treated were given a single dose of one of the following treatments: (i) mannitol citrate buffer (100 µL), (ii) Pluronic F127 MNPs (86.76 mg MNPs/kg, 100 µL/25 g mouse), (iii) PTX-MNPs (6.97 mg PTX/kg, 86.76 mg MNPs/kg), (iv) DOX-MNPs (8.22 mg DOX/kg, 86.76 mg MNPs/kg), (v) Doxil (8.22 mg/kg), and (vi) PTX-Chremophor (6.97 mg/kg). In a dual dose study, mice were treated when tumors reached 300 – 400 mm$^3$ and again 7 days later with one of the following treatments: (i) mannitol citrate buffer (100 µL), (ii) PTX-MNPs (6.97 mg PTX/kg, 86.76 mg MNPs/kg), (iii) PTX-Chremophor (6.97 mg/kg), or (iv) MNPs (86.76 mg/kg). Further controls included a single dose treatment of a Pluronic F127 emulsion in mannitol citrate buffer (13.36 mg/kg), or OA + Pluronic (12.56 mg OA/kg, 13.36 mg Pluronic F127/kg). Six mice were treated in each group with a primary endpoint of tumor volume reaching 10% of the total body weight (detailed below). Exceptions include Doxil and dual dose F127 MNP treatment. Only 3 mice were treated with Doxil due to severe weight loss and side effects of the drug. Additionally, of the six mice from the dual dose F127 MNP treatment group, three of the mice showed signs of skin issues and open wounds likely a result of *Corynbacterium bovis* about 100 days post-treatment and were euthanized.

For the above experiments, all mice were injected intravenously with a dose of 100 µL/ 25 g body weight. The tumor length (mm) and width (mm) were measured every other day and the tumor volume (mm$^3$) was calculated according to the formula in Section 3.2.2.3 (Page 49).
As a parameter of a possible *in vivo* toxicity of the treatments, body weights were monitored every other day. Mice were euthanized with a 100 µL intraperitoneal injection of Sodium Pentobarbital when the tumor volume reached 10% of the body weight according to the following calculation:

\[
\%\text{BodyWeight} = \frac{TumorVolume}{BodyWeight \times 1000 - TumorVolume} \times 100
\]

Mice that showed severe weight loss (greater than 10% of the initial body weight) at any time during the course of the experiments were euthanized, necropsies were performed, and any tissue that was abnormal was harvested and sent for a pathohistologic examination (results not shown). These mice were found to have cervical hyperplasia and urinary bladder distension with urine retention caused by the exogenous estrogen. These mice were excluded from the study.

5.2.3 Blood Flow Perfusion

The BFP measurements were carried out using an ADInstruments Blood Flow Meter. The equipment uses an infrared laser to determine the flow rate of red blood cells using the Doppler method. Readings were taken using an ADInstruments OxyFlo Probe to measure blood flow non-invasively in athymic nude mice. Mice were anesthetized with isoflurane before being measured. Readings were taken for 45 seconds at the site of the tumor and just behind the head, on the back of the neck. Measurements were done using a 50Hz low-pass filter, and a signal of 5mV was converted to an arbitrary measurement of 1 Blood Perfusion Unit (BPU). The average BPU measurement for each
45 second reading was recorded. Blood flow was monitored when tumors reached a size of 250 mm$^3$ and daily measurements were taking until seven days post treatment. Mice were treated with saline (100 µL/25 g mouse) or F127 MNPs (86.76 mg MNPs/kg mouse, 100 µL/25 g mouse) when MCF7 tumor size reached 300 mm$^3$.

5.2.4 Lipid Peroxidation

When MCF7 tumor size reached 300-400 mm$^3$ mice were treated with saline (100 µL/25 g mouse) or F127 MNPs (86.76 mg MNPs/kg mouse, 100 µL/25 g mouse). The malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) generated by lipid peroxides was used to detect lipid peroxidation ex vivo. At 1, 2, and 4 days post saline or MNP injection mice were euthanized and a necropsy was performed to extract the tumor, heart, lungs, liver, spleen, and kidneys, snap frozen in liquid nitrogen and stored at -80 ºC until analysis. The organs were homogenized in 20 mM PBS on ice (10 mL PBS/g tissue) and then analyzed for lipid peroxidation using the Oxis Research LPO-586 Colorimetric Assay for Lipid Peroxidation (Burlingame, CA). Briefly, 1 mL of the homogenized samples were centrifuged at 8,000 rpm for 10 min at 4 ºC. A 50 µL sample of the supernatant or standards (0-20 µM MDA) were combined with 167.5 µL of R1 reagent and 37.5 µL of the R2 reagent. The samples were vortexed and heated for 1 h at 45 ºC, then centrifuged at 13,000 rpm for 10 min at 4 ºC. The supernatant was immediately transferred to a 96 well plate and absorbance analyzed at 586 nm. A wavelength scan was performed to ensure that the reading was due to the MDA/HAE reaction at 586 nm, and not the tail-end of the peak of a non-specific reaction at 500 nm.
5.2.5 Apoptosis

When MCF7 tumor size reached 300-400 mm$^3$ mice were treated with saline (100 µL/25 g mouse) or F127 MNPs (86.76 mg MNPs/ kg mouse, 100 µL/25 g mouse). Seven days post-treatment the mice were euthanized and perfused with cold saline. The tumor, heart, lung, liver, kidney, spleen, and brain were then extracted and fresh frozen in OCT Compound. The embedded tissues were then cryo-sectioned into 5-16 µm sections and fixed to glass slides. The slides were then stained for apoptosis using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit.

Images of histological cross-sections were acquired using a Leica DMR upright microscope (Heidelberg, Germany), a 10X objective, mono filter, and a Retiga 2000R CCD digital camera (Q-Imaging, Burnaby, B.C., Canada). Image acquisition was fully automated using an 8-slide, linearly-encoded X, Y, Z-motorized stage (Prior Scientific, Rockland, MA) managed by Objective Imaging’s Oasis 4i controller (Kansasville, MI), TurboScan software (Objective Imaging) and Image-Pro 6.2 (Media Cybernetics, Silver Spring, MD). During acquisition, high magnification (auto-focused) image fields were acquired across the entire tissue section and stitched together to form a single high resolution, large field-of-view (0.74 µm/pixel).

Three sections of each organ were cut, stained, and imaged using this procedure for each mouse. The average percent apoptosis for each mouse was then used in a 1-tailed t-test to determine significance (n =4, α = 0.05).
5.2.6 Reactive Oxygen Species In Vitro

Reactive oxygen species were measured in vitro with the chemiluminescent probe L-012 (Wako Chemicals USA, Richmond, VA). Phosphate buffered saline (2 mL) was added to a disposable cuvette with 2.5 µL L-012 (1 mg/mL final concentration 1.25 µg/mL). The sample was placed in a TD-20/20 Luminometer (Turner Designs, Fisher Scientific). Samples of H$_2$O$_2$ (7.75 µL, 50 µM final concentration) and 4 µL Fe$^{2+}$ (5 µM Fe$^{2+}$ final concentration from Ammonium Iron II sulfate hexahydrate) were added simultaneously to the cuvette. The luminometer readings had a 0 s delay with a 5 s integration time for 20 consecutive runs. Samples of H$_2$O$_2$ (50 µM), Fe$^{2+}$ (5 µM), H$_2$O$_2$ (50 µM) + MNPs (5 µM Fe$^{2+}$) and H$_2$O$_2$ (50 µM) + MNPs (50 µM Fe$^{2+}$) were used as controls.

5.2.7 Detection of Reactive Oxygen Species In Vivo

When MCF7 tumor size reached 300-400 mm$^3$ mice were treated with saline (100 µL/25 g mouse) or F127 MNPs (86.76 mg MNPs/kg mouse, 100 µL/25 g mouse). The chemiluminescence from the probe L-012 was used to detect ROS in vivo with the Xenogen IVIS 100 (Caliper Life Sciences, Hopkinton, MA) imaging system. Twenty-four hours post saline or MNP injection, mice were injected intraperitoneally with L-012 in water (25 mg/kg, 100 µL for a 25 g mouse). Mice were imaged 10 min post L-012 injection. Regions of interest were manually drawn over the tumor and a small sample of muscle in the hind limb to determine the amount of ROS generated (given as radiance, p/s/cm$^2$/sr).
### 5.2.8 Statistical Analysis

To compare the tumor growth over time between groups of mice, the following area under the curve (AUC) formula was used to calculate the total tumor volume for each mouse:

\[
AUC = \sum_{i=1}^{n} \frac{1}{2} (t_{i+1} - t_{i})(v_{i+1} + v_{i})
\]

where \( t \) is the time in days, \( v \) is the tumor volume in mm\(^3\), and \( n \) is the day at which the first mouse was euthanized (defined above). Graphed data are the means ± SEM. Statistical significance was determined by ANOVA. The Kaplan-Meier method was used to calculate survival function, and the differences were assessed with the log-rank statistic.

### 5.3 Results

The size of our MNPs after loading with Doxorubicin (DOX-MNPs) and Paclitaxel (PTX-MNPs) is similar to the plain MNPs at ~220 nm (Table 5.1, page 92). Doxorubicin, because it is cationic, resulted in the MNPs having a more positive charge. The charge of PTX-MNPs was similar to the plain MNPs. To determine whether our MNPs provide sustained drug release and tumor regression \textit{in vivo}, we treated mice bearing MCF-7 breast xenograft tumors with a single dose of DOX-MNPs or PTX-MNPs via tail vein injection when the tumors reached ~300 mm\(^3\). Control mice were treated with either mannitol citrate buffer or plain MNPs. There was a significant difference in the tumor growth between these groups, and we observed decreased tumor growth in both drug loaded MNP groups and the plain MNP group compared to the mannitol citrate
Table 5.1 MNP Drug Loading Characteristics

<table>
<thead>
<tr>
<th>MNP Formulation</th>
<th>Size (nm)</th>
<th>PI</th>
<th>Zeta (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F127 MNPs</td>
<td>222.8</td>
<td>0.115</td>
<td>-31.1</td>
</tr>
<tr>
<td>DOX-MNPs</td>
<td>229.2</td>
<td>0.124</td>
<td>-10.1</td>
</tr>
<tr>
<td>PTX-MNPs</td>
<td>214.3</td>
<td>0.124</td>
<td>-33.1</td>
</tr>
</tbody>
</table>
buffer control group (Figure 5.1 A, page 94). Treatment with either drug-loaded or plain MNPs resulted in a significant increase in survival compared to the mannitol citrate buffer control (Figure 5.1 B, page 94).

We next asked whether a dual dose of our PTX-MNPs or plain MNPs would result in further tumor regression and increased survival compared to either control mannitol citrate buffer or a current clinical formulation of Paclitaxel in a Chremophor emulsion, Taxol. We chose a dual dose of Paclitaxel over Doxorubicin because of the toxic side effects of free doxorubicin; when free Doxorubicin was given at the same dose as loaded in the MNP formulation, mice suffered severe weight loss and required euthanasia. Compared to control mannitol citrate buffer or Taxol, we observed a greater decrease in tumor growth in mice treated with our PTX-MNPs and plain MNPs, and prolonged survival in these MNP treatment groups (Figure 5.1 C and D, page 94). In Figure 5.1 E and F, the tumor growth and survival for mice treated with a single dose of plain MNPs is compared to the dual dose given at days 0 and 7 for clarity. Three of the mice received a dual dose of MNPs and were euthanized at 98, 99, and 104 days, but due to skin issues and tail wounds, not due to tumor growth or weight loss as occurred for all of the other mice in the study. Two of the three mice had small tumors \((< 300 \text{ mm}^3)\) while the third mouse did not have a tumor. These results demonstrate that our drug-loaded MNPs effectively ablate tumors and increase survival compared to control mannitol citrate buffer or an equivalent drug dose of the current chemotherapeutic formulations. However, the results raise the question of how our plain MNPs are decreasing tumor growth and increasing animal survival. This result was surprising,
Figure 5.1 Tumor growth and survival rates in mice after magnetic nanoparticle treatment.

Tumor volumes and survival rates following a single (A and B) and dual treatment (C and D) with F127 MNPs in breast tumor-bearing mice. The initial study with a single dose of drug loaded and plain F127 MNPs showed inhibition of tumor growth and significantly improved the survival. These results were confirmed with a dual dose of F127 MNPs (at day 0 and day 7 of treatment) compared with free Paclitaxel in Chremophor and Paclitaxel MNPs. Panels E and F compare single and dual dose with F127 MNP treatment. A) p < 0.01 at 12 days, C) p < 0.0001 at 32 days; ANOVA comparing AUC at first death.
because our plain MNPs did not have any affect on MCF-7 tumor growth when used as a treatment in vitro (Figure 5.2, page 21).39

We generated two hypotheses to explain why our MNPs might be causing tumor regression in vivo, but not in vitro. The first hypothesis was that our MNPs might be obstructing the blood flow in the ill-formed vasculature of the xenograft tumors. Our second hypothesis was that the iron-oxide might be broken down and enter into the Fenton reaction, which produces the toxic hydroxyl radical.

We tested our first hypothesis, that our MNPs might be obstructing blood flow in the ill-formed vasculature of the xenograft tumors, a hypothesis that would explain why our plain MNPs ablate tumors in vivo but not in vitro. Tumor growth and vitality depend on the vasculature and increased blood flow. If the MNPs aggregate and consequently inhibit this blood flow, it could cause the regression of tumor growth. Mice were treated with either saline or our plain MNPs, and the tumor blood flow measured before treatment and daily after treatment with a non-invasive probe placed over the tumor. As a control, the blood flow perfusion was also measured at the base of the neck. The blood flow perfusion meter measures back-scatter and we wanted to ensure that the MNP injection did not interfere with the perfusion measurements. We did not find a significant difference in the blood flow perfusion between MNP treated and saline control mice at the tumor or neck (Figure 5.3, page 97). Based on these results, we concluded that our MNPs were not causing tumor regression by obstructing blood flow.
Figure 5.2 Inhibition of MCF-7 growth in vitro.

MCF-7 cells were treated with free Doxorubicin (DOX), MNPs loaded with Doxorubicin (DOX-MNPs) at varying drug concentrations or an equivalent dose of plain MNPs. Cells were treated with the particles or drug for 24 h and the growth measured after 5 days.

Figure adapted with permission from Jain, et al.\textsuperscript{39} Copyright 2005 American Chemical Society.
Fig. 5.3 Average blood flow perfusion of athymic nude mice monitored over 11 days.

Mice were measured at the tumor site (A) and on the back of their neck (B). Mice were treated with either saline or F127 MNPs on day 4 (Dashed line). Points represent mean ± SEM (n=5).

Percent difference in blood flow perfusion in athymic nude mice from pretreatment at the tumor site (C) and at the back of the neck (D). Points represent mean ± SEM (n=5). A two-tailed t-test was conducted in conjunction with an F-test for the overall treatment. Neither the tumor nor the brain showed statistically different changes from pretreatment in either saline or F127 treatments (α=.10).

Blood flow perfusion at the tumor site represented as a percentage of blood flow to the back of the head (E). Treatment with either saline or F127 MNPs was given on Day 4 (dashed line). Points represents mean ± SEM (n=5).
Free ferrous iron can react with hydrogen peroxide to generate ferric iron and the highly toxic hydroxyl radical in a process known as the Fenton Reaction. We speculated that the iron-oxide core of our MNP might not break down in vitro, but in vivo it might form free iron, thus causing oxidative stress, cell death, and tumor regression in vivo. To confirm that it was the iron component of our MNP and not the Pluronic F127 and Oleic Acid layers, we treated mice with emulsions of either Pluronic F127 or Oleic Acid and Pluronic F127 at the same dose that they were given in the full MNP formulation. The tumor growth and survival for mice treated with these emulsions did not differ significantly from the mannitol citrate buffer control (Figure 5.4 A and B, page 99). The iron-oxide present in our particle is likely responsible for the observed decrease in tumor growth.

Reactive oxygen species are short lived and difficult to measure directly. In order to determine if the iron from our MNPs was causing tumor regression, we looked for the effects of oxidative stress including lipid peroxidation and apoptosis. We also investigated the use of the chemiluminescent probe L-012 for the in vivo detection of ROS. Mice were treated with either saline or mannitol citrate buffer, and their tumors and organs including the heart, lung, liver, spleen, kidneys, and brain were analyzed for lipid peroxidation or apoptosis. There was no change in the lipid peroxidation of the tumor, heart, liver, spleen, or kidneys for the mice treated with MNPs compared to control saline treated mice at days 1, 2 and 4 post-treatment (Figure 5.5 A, page 100). However, there was a significant difference decrease in LPO detected in the lungs of mice treated with MNPs compared to the saline control. This difference was due to non-specific
Figure 5.4 Pluronic and oleic acid alone do not cause tumor regression.

Mice bearing MCF-7 xenograft tumors were injected (tail vein) with emulsions of either Pluronic F127 (concentration) or Pluronic F127 and Oleic Acid (concentrations). The tumor growth was monitored over 40 days (A). Survival for the Pluronic and Pluronic + Oleic Acid treated mice does not differ significantly from the mannitol citrate buffer control (B). A) p = 0.46 at 8 days; ANOVA comparing AUC at first death.
Figure 5.5 Lipid peroxidation in vivo.

A) Mice were treated with either saline or F127 MNPs. At one, two, and four days post-MNP treatment, mice were euthanized, perfused with saline, and their organs analyzed for HAE and MDA. The average concentration in 3-4 organs for each treatment is shown. There was a significant difference in MDA for the lungs of the saline control compared to each of the other organs, however, this difference was confirmed to be non-specific signal at 500 nm not from the MDA/HAE. B) Wavelength scan shows a non-specific peak for all organs at 500 nm (shown is sample spleen). Arrow denotes peak of standard at 586 nm where positive result would appear. Data is average ± SEM; n=3-4, * p<0.05.
interactions of the probe from a peak at 500 nm and not from MDA/HAE at 585 nm; sample in Figure 5.5 B (page 100) shows the non-specific interaction observed in the spleen. Sections of tumors and each of the organs were stained for DNA damage with the TUNEL stain, and the percent of apoptotic cells (those with both TUNEL and DAPI staining overlapping in the nucleus) were quantified. Seven days after treatment, there was a significant increase in the percent of apoptosis in the tumor of mice treated with MNPs compared to saline, but this increase in apoptosis did not occur in any of the other organs (Figure 5.6 A and B, page 102).

The chemiluminescent probe L-012 was used to determine if the hydroxyl radical might be generated. We first tested to see if the probe detected the hydroxyl radical in vitro in the presence of free ferrous iron and hydrogen peroxide. There is a very large generation of the chemiluminescent signal detected in response to the ferrous iron and hydrogen peroxide as a result of the hydroxyl radical production, but no response is detected in the presence of our MNPs at an equivalent or 10 × iron dose, indicating that our plain MNPs are not generating the hydroxyl radical in the presence of hydrogen peroxide or reacting with the probe on their own (Figure 5.7 A, page 103). Free ferrous iron slowly reacts with the probe in the absence of hydrogen peroxide, the signal was ~1.1 relative fluorescence units on the graph at 100 s. MNPs and hydrogen peroxide alone never exceed 0.05 relative fluorescence units, and by 100 s the reaction of hydrogen peroxide with ferrous iron was 1.1 after peaking at 2924 relative fluorescence units at 5 s. The chemiluminescent probe is thus capable of detecting the hydroxyl radical, but does not react with the iron oxide core of our plain MNPs. Further, the
Figure 5.6 Apoptosis in mice treated with saline or F127 MNPs seven days post-treatment.

Mice bearing MCF-7 tumors were treated with either F127 MNPs or saline, and seven days post-treatment their organs were harvested and analyzed for apoptosis with Tunel staining. A) A representative tumor section from a mouse treated with F127 MNPs is shown on the left, and a mouse treated with the control saline is shown on the right. (F127 MNP treatment has 3.5% apoptosis with a mean of 4.25% for F127 MNP treatment; Saline treatment shown has 1.7% apoptosis and the mean for the group was 1.6%; Scale bar = 1 mm). Blue represents DAPI+ cells and green represents FITC+ cells. B) The percentage of apoptosis was calculated as a ratio of FITC+/DAPI+ cells for each organ. Data represented as mean ± SEM (n=4, * p<0.05).
Figure 5.7 Reactive oxygen species detection \textit{in vitro} and \textit{in vivo}.

A) The chemiluminescent probe L-012 detects the hydroxyl radical generated from ferrous iron (Fe$^{2+}$) and H$_2$O$_2$, but not iron-oxide MNPs in the presence of H$_2$O$_2$. Mice were treated with either F127 MNPs (21.69 mg/mL, 100 µL) or Saline when tumors reached ~300 mm$^3$. One day later, and each day for the next two weeks, mice were injected with the L-012 (6.25 mg/mL in water, IP) and imaged with the Xenogen IVIS 100 after 10 min. B) Chemiluminescence is detected \textit{in vivo} in mice injected with the probe L-012 at high concentrations in the intestines, and also in the tumors of mice (circled in red). C) Tumor growth in mice treated with F127 MNPs is significantly decreased compared to mice treated with saline (n=7, * p<0.001). Daily chemiluminescence for two weeks in the tumor (D), a small portion of the muscle on the right hind limb (E) or a region of interest surrounding the intestines (F). Data is average ± SEM; n=7, * p<0.05.
production of signal with iron alone is much slower and likely will not considering the probe will first react with the rapidly produced hydroxyl radical.

To determine if the hydroxyl radical was being generated \textit{in vivo}, the chemiluminescent probe was injected intraperitoneally in mice treated with plain MNPs or saline beginning the day after treatment and daily for the next 14 days (Figure 5.7 B, page 103). The tumor growth was measured daily and we see a significantly slower growth in the tumors of mice treated with F127 MNPs and compared to saline days (Figure 5.7 C, page 103) indicating that the probe was not interfering with the treatment. The chemiluminescent signal detected with the IVIS 100 \textit{in vivo} imaging system shows strong signal in the intestines, and a significant increase in signal in the tumors of mice the day after treatment, but not in the subsequent 13 days (Figure 5.7 D). There was no difference in the signals between the MNP treated or saline treated mice in a small section of the muscle on the right hind limb, or in a region of interest drawn over the intestines on any of the 14 days post-treatment (Figure 5.7 E and F).

5.4 Discussion

Our experiments show that the drug-loaded MNPs can reduce tumor growth and increase survival as compared to saline control in mice bearing MCF7 breast xenograft tumors. A surprise finding was that our plain MNPs also reduced tumor growth and increased survival, which was better than the drug-loaded MNPs. The effect of control MNPs may be caused by iron in the formulation generating oxidative stress \textit{via} the Fenton Reaction and ablating the tumors in the absence of traditional chemotherapeutic
agents.

Iron-oxide nanoparticle formulations are typically developed as tumor contrast agents for magnetic resonance imaging, for magnetic hyperthermia, and for magnetic drug targeting. Few nanoparticles with multifunctional applications for imaging and cancer therapy have been tested in vivo. A limited number of formulations have examined the effects of iron-oxide based nanoparticles on tumor growth and survival in vivo, and interestingly the studies both support, and conflict with our finding that iron-oxide nanoparticles themselves ablate tumors.\textsuperscript{37, 106-108} An iron-oxide nanoparticle formulation designed to ablate tumors with magnetic hyperthermia increases the anti-tumor activity in the absence of the hyperthermia, though not significantly from the control.\textsuperscript{37, 106} The authors speculate that the iron from the formulation is generating oxidative stress as the particles are broken down.\textsuperscript{37, 106} In a glioblastoma model that used convection enhanced delivery to bypass the blood brain barrier, both the targeted treatment of EGFRvIIIAb iron-oxide nanoparticles and the control iron-oxide nanoparticles showed a significant increase in survival (median survival 19 and 16 days, respectively), compared to the HBSS control (median survival 11 days).\textsuperscript{107} Conversely, a HER2 targeted MNP is only effective in treating mouse the mouse fibroblast NIH3T6.7 tumors in combination with the drug Doxorubicin, while control particles that do not contain drug have a growth pattern similar to the untreated animals.\textsuperscript{108} The observation that their plain nanoparticles do not inhibit tumor growth may be in part due to the cell type, as a separate study treating the mouse fibroblast cell line L929 show iron does not generate a toxic effect.\textsuperscript{109}
Reasons for the apparent discrepancies between various iron-oxide based nanoparticles inducing tumor regression may be a result of the dose of iron delivered with the particles, the route of delivery, or the formulation coating. In a single nanoparticle injection, we deliver ~1.1 mg iron in the ~2.2 mg injected dose. In the studies conducted by Balivada, where a slight inhibition of tumor growth was observed with plain iron-oxide nanoparticles, and Hadjipanayis, where similar survival was observed without targeting, the amount of iron injected were, respectively, 360 µg (3 intratumoral injections over 3 days) and 2 µg (convection enhanced delivery to the brain). Each of these formulations inject far less iron with the nanoparticles, however, the delivery is directed to the tumor either with direct injection or bypasses the RES with direct injection to the brain. HER2 MNPs, which did not alter tumor growth compared to the control, were injected intravenously but with ~120 µg – 10% of the total iron we injected with our formulation. Direct delivery of iron-nanoparticles to the tumor may allow a smaller dose to demonstrate anti-tumor effect, while IV injection may require a higher iron-dose due to the larger volume of distribution and clearance of the nanoparticles by the liver and other organs of the RES.

The promising effect of iron-oxide nanoparticles as a treatment for cancer may be due in large part to the dose of iron delivered during the study. Our nanoparticle dose is injected in a single dose of ~1.1 mg Fe/25 g mouse, or ~ 44 mg Fe/kg. In clinical and pre-clinical trials where iron based formulations are used for imaging, the doses recommended for iron-oxide nanoparticles range from 2.6-4 mg Fe/kg, though in some cases the authors speculate that an increased dose would be more desirable to improve
contrast and diagnosis.\textsuperscript{30} When using iron-oxide formulations such as ferumoxytol in the anemia setting two doses of 1000 mg of ferumoxytol, corresponding to 14.3 mg Fe/kg in a 70 kg person can be administered with few side effects.\textsuperscript{30} Additionally, there are some clinical situations that would require up to 50 mg/kg iron replacement to be given, though these therapies are currently given at a maximum of 20 mg/kg due to dose limiting toxicities.\textsuperscript{110, 111} The side effects that typically exclude patients from these studies include hypersensitivity to parenteral iron or the dextran coatings associated with the particles. Thus, if the coating in our present formulation leads to reduced hypersensitivity from parenteral injections of the formulations, our formulation may be a promising option for increasing the iron-dose and leading to an iron-induced cytotoxicity specifically targeted to tumor cells.

A final reason for the differences in effect of iron-oxide nanoparticles on tumor growth may be the formulation coating. Where plain MNPs decreased tumor growth, the iron-oxide was anchored with stealth organic glycol ligands or amphiphilic block copolymers similar to out MNP formulation. Where no effect of plain MNPs on tumor growth was observed, the nanoparticles consisted of magnet-polymeric nanohybrid consisting of a magnetic nanocrystal then coated with an amphiphilic block copolymer. The breakdown and clearance of these particles \textit{in vivo} may influence whether or not the iron can affect the tumor growth.

An additional noteworthy observation with our MNP formulation is that it is non-toxic \textit{in vitro}, but there is selective toxicity to cancer cells \textit{in vivo} and a decrease in tumor
growth compared to the control saline. This contrasts the results observed with the antianemic iron therapy iron-sorbitol-citrate, which ablates a variety of cancerous cells but not normal cells in vitro, and inhibits murine melanoma B16 cells in vivo.\textsuperscript{109, 112} The differences in the formulation of the iron may be why these particles have been identified to ablate cancer cells in vitro while iron-oxide nanoparticles are typically considered to be non-toxic in vitro. Iron-oxide nanoparticles must first be broken down before entering into the Fenton reaction, which may not happen in cell culture. On the other hand, the iron replacement therapies contain ferric iron, which may more readily interact to inhibit cell growth or be more easily converted to ferrous iron in vitro and enter into the Fenton reaction.

MNPs can be taken up by cancerous cells as well as cells of the tumor stroma, and tumors appear to recycle iron from the local environment for their own use.\textsuperscript{113} Macrophages in particular can take up iron and, under conditions of severe lysosomal iron overload, are more susceptible to cytotoxicity from H$_2$O$_2$. Disrupting the surrounding microenvironment and iron stores of the tumor may affect tumor proliferation. To determine if the surrounding cells are involved in the tumors iron homeostasis, tumor sections could be analyzed for markers of intracellular iron storage and for localization within tumor cells and macrophages. Such results might indicate if surrounding cells could contribute to bystander mechanisms of action of MNPs. Additionally, more severely immunocompromised mice, such as SCID mice could be inoculated with tumors and treated with MNPs. Altered patterns in tumor growth and survival compared to athymic nude mice might indicate if an innate immune response,
such as natural killer cells, contributes to the altered tumor growth and increase survival we observe with our MNP treatment.

One reason that drug-loaded MNPs may not be as effective as plain MNPs may be that there is a change in the biodistribution of the MNPs. After drug loading, DOX-MNPs are more positive in charge than plain MNPs (-10 vs -30 mV, respectively). The more positive charge may result in interactions with different proteins and opsonins leading to more rapid clearance of the drug loaded MNPs and less MNPs and drug reaching the tumors. However, the above explanation does not hold true for PTX-MNPs, which do not show significant change in surface properties following drug loading (Table 5.1, page 92). Another possibility could be that after loading with drug the PPO subunit of the MNPs may not anchor as strongly onto the OA layer, leading to more rapid dissociation and thus clearance in vivo compared to plain MNPs, and less MNPs and drug reaching the tumor. We were able to track the long-term biodistribution of plain MNPs with fluorescent dyes; simultaneously loading MNPs with dyes (0.5% w/w) and drug (10% w/w) could show if the drug alters the biodistribution of the MNPs.

Another option that drug loaded MNPs may be less effective than plain MNPs may be because of the order in which the cancer cells are exposed to the drug and MNPs. Both DOX and PTX may be released before the iron-oxide nanoparticles are broken down. This release may slow the uptake of additional MNPs, or slow the growth of cells leading to less uptake of MNPs and potentially less oxidative stress generated from the plain MNPs.
A final reason that drug loaded MNPs could be less effective than plain MNPs may be caused by a combination of the MNPs generating oxidative stress and the chemotherapeutic agents acting as autophagy-inducing agents leading to tumor cell survival. Autophagy is generally known as an alternative cell death pathway, however, in times of stress it may act to promote cell survival by removing damaged proteins and organelles, leading to increased tumor growth.\textsuperscript{114-115} Doxorubicin and Paclitaxel induce apoptotic cell death, but they also utilize autophagy as a cell survival strategy.\textsuperscript{116-117} Low levels of doxorubicin mainly result in activation of autophagy rather than apoptosis.\textsuperscript{118} Thus, our nanoparticles, which provide sustained release of the drug over time, may be releasing the drug at a level that promotes autophagy. By inducing autophagy in the oxidatively stressed cancer cell, the drugs may be antagonizing the oxidative kill effect and promoting tumor cell survival. The use of hydroxychloroquine, a clinically approved anti-malarial and known inhibitor of autophagy, in combination with Doxorubicin or Paclitaxel supports this theory. Hydroxychloroquine prevents autophagy and promotes apoptotic cell death in combination with Doxorubicin, and is being investigated in clinical trials as an adjuvant therapy with Paclitaxel for improved treatment of non-small cell lung cancers.\textsuperscript{118-119} Hydroxychloroquine could thus be used as an adjuvant therapy with our drug-loaded MNPs to inhibit autophagy, and may result in a synergistic effect where the chemotherapeutic agents with the MNPs are a more powerful combination for the treatment of tumors than the plain MNPs themselves.

Thus there are multiple possibilities for reduced efficacy with drug-loaded MNPs compared to control MNPs, further studies are required to explain the outcome.
Nonetheless, our MNPs without drug did not cause any weight loss and animals appear otherwise healthy. Hence, tumor growth inhibition seen in our studies with control MNPs is not due to a toxic effect in general; there appears to be a specific effect on tumors that is responsible for suppressing the tumor growth. Further studies are required to understand the mechanism of tumor growth inhibition with control iron-oxide MNPs and to explore their application for cancer therapy.
CHAPTER 6: SUMMARY, CLINICAL SIGNIFICANCE, AND CONCLUSIONS

6.1 Summary

The aims of this thesis were to evaluate MNPs as a platform for magnetic resonance and optical imaging, investigate the role of magnetic field targeting on MNP biodistribution and tumor targeting, and evaluate their efficacy in treating breast tumors \textit{in vivo}. It was hypothesized that the multifunctional MNP, with applications in imaging and drug therapy, would enhance tumor identification, targeted drug delivery, and tumor response to therapy.

We found that our F127-modified MNPs provided sustained and enhanced contrast MRI contrast throughout the tumor mass, whereas contrast was transient and confined to the tumor periphery with Feridex IV and our T908-modified MNP. The Oleic acid layer provided a platform to load hydrophobic NIR dyes for optical imaging and longitudinal tracking of the MNPs. The particles reached a peak localization in the tumor two days post-injection, and were cleared over the next twelve days. With exposure to a one-hour magnetic field, the MNPs had increased localization within the tumors 24 h after the removal of the magnet. With the option of loading spectrally distinct fluorophores into the MNPs and ligand targeting, it may be possible to evaluate how formulation characteristics (such as the interplay of physical characteristics and targeting ligands) may increase the accumulation of MNPs within tumors and eventually enhance therapeutic efficacy. While the MNP was designed as a drug delivery platform and determined to be non-toxic \textit{in vitro}, we found that our plain MNPs were an effective
cancer therapy in vivo without toxicity to healthy organs. The reasons for the selective toxicity of MNPs and their potential role in generating oxidative stress as a means of tumor ablation are explored below.

6.2 An Underappreciated Role of Iron-Oxide Nanoparticles for Cancer Therapy

Iron-oxide nanoparticles facilitate cancer diagnosis through enhanced contrast, selectively enhance tumor cell death with magnetic hyperthermia, and improve drug delivery with magnetic drug targeting. One application that remains largely unexplored is using the iron-oxide nanoparticles themselves to selectively inhibit tumor growth. Based on the findings in Chapter 5 of this dissertation, we propose that high doses of iron-oxide nanoparticles can be used as a treatment for cancer by generating an oxidative assault against cancer. This proposal may be met with resistance considering the controversy surrounding iron in the field of cancer. Iron generates reactive oxygen species through the Fenton reaction, which may both cause – or cure cancer. Additionally, high demand for iron by cancer cells leads to contradictory therapeutic approaches: iron deprivation or overdose are both potential cancer therapies. A schematic connecting the role of iron in cancer and nanoparticle applications is shown in Figure 6.1, page 114.

6.2.1 A Cause and a Cure: Iron Generated Oxidative Stress

Chronic oxidative stress is one of the mechanisms by which malignant transformation occurs; however, selective induction of acutely high levels of oxidative stress in tumor tissue has the potential to destroy and/or arrest the growth of cancer cells. Iron is toxic in its elemental state; free ferrous iron (Fe^{2+}) can participate in the
Iron can generate reactive oxygen species (ROS) through the Fenton reaction, leading to DNA damage and carcinogenesis in healthy cells, or contribute to cell death in tumors. Cancer cells sequester iron to aid in proliferation; removing this essential nutrient with iron chelators or overdosing cells with iron to cause oxidative damage are two mechanisms to inhibit cancer progression. Iron based nanoparticles may increase intratumoral iron and generate an oxidative assault leading to tumor regression. Nanoparticles have further multifunctional applications in cancer diagnosis (magnetic resonance imaging and detection of circulating tumor cells) and therapy through magnetic hyperthermia and magnetic drug targeting.
Fenton reaction, producing the highly toxic hydroxyl radical (•OH). Increased iron consumption or high body iron stores may lead to increased incidence of a variety of cancers in humans through increased oxidative stress.\textsuperscript{122-125} Thus, because of its known ability to generate DNA damaging hydroxyl radicals and potentially cause cancer, the potential to have free iron in the body is generally a cause for concern.

On the other hand, cancer cells are generally deficient in the antioxidant enzymes present in normal cells,\textsuperscript{126} making them particularly vulnerable to an oxidative assault. The metabolic demands of cancer cells manifest in increased levels of transferrin receptors to facilitate iron uptake, and decreased levels of ferroportin (an iron export protein) to control iron homeostasis compared to normal cells.\textsuperscript{127} The differences in iron metabolism between cancer cells and normal cells suggest an iron-mediated oxidative assault may be a mechanism for selectively targeting cancer cells while leaving normal cells unharmed.\textsuperscript{126}

\textbf{6.2.2 Manipulating Tumor Iron Stores for Cancer Therapy}

Disrupting tumor iron stores through iron depletion or iron overload results in growth arrest. One potential therapeutic approach in cancer cell manipulation is to rob cancer cells of this essential nutrient with iron chelators. Desferal (desferrioxamine mesylate) is clinically approved for the treatment of iron overload as an anti-cancer therapeutic in animal models and clinical trials.\textsuperscript{128-129} Iron chelators cause growth arrest in some tumors, though their real utility may be in the use as a combination therapy with Doxorubicin to prevent the iron mediated cardiotoxicity associated with administration of
Because of the limited success of iron chelation in cancer therapy and a need for more potent chelators to be developed, and based on our findings that MNPs cause tumor regression and increase survival, we propose that manipulating iron stores in the other direction, i.e. causing iron overload, will be a more successful therapeutic approach for the treatment of cancer.

The idea that iron-overload can selectively inhibit tumor growth holds true in practice: in an aggressive T-cell lymphoma, blocked production of the iron storage protein ferritin heavy chain led to an increase in the free intracellular iron stores, ROS, and resulted in decreased tumor growth while normal cells were unharmed. The antianemic iron therapy Ferric-Sorbitol-Citrate ablates a variety of cancerous cells but not normal cells in vitro, and inhibits murine melanoma B16 cells in vivo. The idea then, that iron overload can be used to selectively inhibit cancer growth while leaving normal cells unharmed has been recognized, but the best way to achieve this result remains largely unexplored.

6.2.3 Concluding Remarks of Iron for Cancer Therapy

The altered regulation of iron in cancerous cells compared to normal cells, along with the potential for iron mis-regulation to selectively cause oxidative stress and cell death makes iron overload an attractive therapeutic idea for the treatment of cancers. We propose that high doses of iron-oxide nanoparticles may be used to generate an oxidative assault for cancer therapy. Compared to iron replacement formulations as a therapeutic agent, the MNPs will offer multifunctional applications for the diagnosis, combined
therapeutic potential, and monitoring of progression of the disease. Further, the iron in MNPs is not stored in its elemental form, which may reduce some of the associated toxicity. There may be dose dependent effects of iron required for tumor ablation. Whether ablation is achieved through a single high dose, or can be achieved with multiple smaller doses remains to be elucidated. Higher doses may be better achieved through development of new formulations, which could have fewer adverse drug events than current iron therapies.

As a supplement to current treatments where iron-oxide nanoparticles will be used in diagnosis or therapy with magnetic hyperthermia and drug delivery, iron based formulations may boost the local oxidative stress and enhance the tumor cell death. With the development of many multifunctional formulations, challenges include achieving optimal tumor kill from each aspect of the formulation, without compromising the activity of the other applications. For combined iron-drug therapy, the drug administered, or even the order - before, with, or after - magnetic nanoparticle injection may drastically alter the activity as was observed with combined cisplatin/hyperthermia therapy.\textsuperscript{50} While MNPs localize to the tumors passively through the EPR effect, a targeted formulation may further increase the effectiveness of the proposed therapy. Local delivery of the iron-oxide through targeting by a magnetic field or through receptors over-expressed on the cell surface should be explored.

The potential for iron to generate cancer, and a past history of sarcoma formation in rats and in a few cases in humans may lead to resistance of the idea of using MNPs as
a tumor therapy.\textsuperscript{131-134} However, the cellular nature of cancer, its increased need for iron, ability to sequester iron, inability to expel iron, and general elevated levels of oxidative stress could make iron an ideal therapy.

6.3 Final Conclusions

The cure to cancer has remained an elusive goal despite our best efforts for earlier diagnosis and improved surgical, chemotherapeutic, and radiation therapies. Advances in the chemotherapeutic delivery in the form of targeted immunotherapeutics, liposomal and albumin bound delivery vehicles have been successfully translated to the clinic in only a limited number of instances. Nanoparticle contrast agents applied to the detection of cancer have been met with only slightly better success compared to drug delivery vehicles in translating the nanoparticle technologies to the clinic. The inherent nature of iron-oxide nanoparticles offers many advantages: contrast agents for the detection of cancer, combination with therapeutic agents for imaging and drug delivery applications, active targeting potential with conjugation to tumor specific ligands or through magnetic drug targeting, and the potential to generate oxidative stress resulting in selective tumor kill.

There is no one treatment for cancer, but the versatility of a multifunctional particle would allow the nanoparticle to be used to diagnose, then aid in the surgical resection followed by drug delivery. Iron based particles have further potential to be used for magnetic hyperthermia and drug delivery to improve outcomes after completion of the surgery or hyperthermia treatment. The wide range of applications, from diagnosis, to
magnetic targeted drug delivery, hyperthermia and stimulus response of drugs will likely make multifunctional MNPs one of the most promising candidates for multifunctional nanoparticle translation to clinical practice.

6.4 Acknowledgments

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REFERENCES


