Magnetic Nanoparticles in the Prevention of Neointimal Hyperplasia

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MAGNETIC NANOPARTICLES IN THE PREVENTION OF NEOINTIMAL HYPERPLASIA

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Elliott Mappus
May 2015

Accepted by:
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ABSTRACT

The use of vascular stents to treat occluded blood vessels is common practice; however, this procedure is often complicated by neointimal hyperplasia reocclusion and thrombogenesis. One treatment option is systematically administering heparin to activate antithrombin III leading to deactivation of thrombin and other proteases involved in blood clotting. This treatment is associated with high rates of bleeding and other vascular complications. In addition to the widely known anti-coagulation effects, heparin has long been known to exhibit an anti-proliferative effect on the growth of cells. The ideal solution would be localized delivery to the site of the interest. Recently, the advancements in magnetic resonance have allowed magnetic nanoparticles to be localized at sites of interest. We propose that a heparin-coated magnetite nanoparticle will fit this ideal solution given it’s potential to deliver localized anti-coagulation and anti-proliferative effects. In this study, we present the synthesis, characterization, and initial cytotoxicity studies of such a particle.

Magnetite nanoparticles were synthesized and characterized to determine magnetic core diameter, hydrodynamic diameter, zeta potential, and heparin loading. Live/Dead and MTS assays were utilized to assess cellular toxicity on vascular smooth muscle cells (VSMCs). Cellular uptake and actin distribution of VSMCs post nanoparticle treatment was observed with Prussian Blue Staining and immunofluorescence respectively.
Nanoparticles were characterized by TEM to be in the middle of our target range with a diameter of 24.3 nm. Heparin loading was found to range from 0.976 to 2.8896 µg heparin/µg nanoparticle depending on the synthesis batch. Proliferation and cytotoxicity studies on vascular smooth muscle cells showed that at the low loading of heparin on nanoparticles, there is indication of proliferation inhibition without VSMC cell death. There was not a noticeable cellular uptake of heparin nanoparticles; however, actin distribution gives possible indication that VSMCs were induced into their contractile phenotype. The results from this study demonstrate a successful synthesis route of heparin-coated nanoparticles and indications for further investigation of VSMC response.
DEDICATION

This work is dedicated to my family and friends. They have stood by me through the highs and lows, and I would not have been able to complete this piece without your support. The unwavering encouragement has sustained me through this research. I especially would like to thank my parents, Jackie and Stephen, for teaching me the importance of education and that there is nothing you can’t achieve if you work hard.
I would like to thank my advisor, Dr. Delphine Dean, for her unwavering support and guidance throughout my research. She has been a positive role model, and I am very appreciative of all of her many lessons that will impact the rest of my career. I would also like to thank my committee members, Dr. Martine LaBerge and Dr. O. Thompson Mefford, whose instruction helped guide my research. I would like to thank the laboratories of Dr. Bruce Z. Gao and Dr. Martine LaBerge for the harvesting and isolation of cells used in this work. I would like to thank the laboratory of Dr. O. Thompson Mefford, especially Benjamin Fellows and Katie Davis, for help with nanoparticle synthesis. I wish to acknowledge the members of my lab, especially Dr. Jorge Rodriguez, Aesha Desai, and Tyler Havey, for assistance with various aspects of the project.
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CHAPTER ONE
INTRODUCTION

1.1 Motivation

The invention of the angioplasty endovascular procedure revolutionized the treatment of arterial disease. Unfortunately, this novel treatment was accompanied by a new pathology, restenosis, which is the reocclusion of the previously cleared vessel. Restenosis is caused by thrombosis and neointimal hyperplasia, the excessive proliferation of vascular smooth muscle cells (VSMCs). Treatment options are limited to systemic administration of therapeutics or targeted drug delivery through drug-eluting stents, with the latter option being the most popular due negative side from systemic administration of the drugs. Recently nanotechnology has offered a new drug delivery system in the form of magnetic nanoparticles capable of being controlled by a magnetic field.

The purpose of this research was to determine if heparin, a naturally occurring proteoglycan with anticoagulation and antiproliferation properties, has the potential to be used in targeted drug delivery via magnetic nanoparticles. Specifically, we aim to synthesize a biocompatible nanoparticle with an outer heparin coating. After characterizing the properties of the heparin nanoparticle, we performed a series of proliferation and toxicity studies to evaluate the feasibility of such a particle in the prevention of neointimal hyperplasia.
1.2 Research Aims

Aim 1: Synthesize and Characterize a Heparin Coated Magnetic Nanoparticle

Biomedical applications of magnetic nanoparticles have very specific size requirements to achieve optimal biocompatibility while maintaining the magnetic properties that make them desirable. For our pilot study, we synthesized 25 nm magnetite nanoparticles and stabilized them with a poly (ethylene glycol) polymer. The nanoparticles were functionalized with low molecular weight heparin. Nanoparticle characterization included measurement of the magnetic core, the hydrodynamic diameter, the zeta potential, and quantification of heparin loading.

Aim 2: Evaluate the Proliferation and Toxicity of Heparin Coated Nanoparticle on VSMCs

After successful synthesis of heparin coating magnetic nanoparticles, their impact on VSMCs was evaluated. We observed the proliferation and toxicity of the particles at early stages in treatment. Cellular uptake of particles was observed to estimate the fate of the nanoparticle. Inhibition of VSMCs is often accompanied by a phenotypic change; therefore, we observed the distribution of α-smooth muscle actin.

1.3 Significance

This study describes the first attempt to utilize the natural proliferation inhibitor, heparin, bound to a magnetic delivery system for treatment of neointimal hyperplasia. The results of this study are indicative for future investigation to determine optimal
heparin loading and dosing concentrations. The benefit of such a therapy could change the way neointimal hyperplasia is treated. Current treatment methods have major drawbacks such as negative systemic side effects and limited amount of deliverable drug. Magnetic nanoparticles offer a means to deliver as much therapeutic as needed and keep the drug localized at the target of interest. The use of naturally occurring heparin has the advantage over most synthetic drugs with dual effects in anticoagulation and antiproliferation. Such a treatment option is much needed in treating neointimal hyperplasia.
2.1 Introduction

The use of nanoparticles, particularly magnetic nanoparticles, has become a topic of great interest for biomedical researchers. Researchers have developed synthesis methods allowing the creation of particles with vast range of diameter sizes. Due to their size, nanoparticles are able to interact at the cellular and molecular level of biological interactions [9]. Magnetic nanoparticles have another advantage in that they obey Coulomb’s law allowing manipulation by an external magnetic field. There exist a number of potential industrial uses for magnetic nanoparticles including magnetic ink for bank checks, magnetic seals in motors, data storage, and biomedical applications [5]. Biomedical applications typically require the nanoparticle to have superparamagnetic behavior at room temperature, which is characterized by no overall remains of magnetism due to a rapidly fluctuating magnetic state [3]. Additionally, the nanoparticle must be stable in aqueous solutions at a neutral pH with physiologic salt concentrations.

Nanoparticle stability is of upmost importance for biomedical applications; therefore, particle size is also of crucial importance. Gravitational forces naturally cause nanoparticles to attract and precipitate out of solutions; therefore a stabilization method is often employed. Two common methods for stabilization are steric and columbic interactions [3]. Steric stabilization methods with a biocompatible polymer are preferred for \textit{in vivo} applications as they hinder biodegradation in addition to preventing
nanoparticle aggregation [11]. Particles that meet the above requirements have potential for many biomedical applications. In this literature review, we will discuss the applications of magnetic separation, targeted hyperthermia, magnetic resonance imaging (MRI) contrast agents, and targeted drug delivery.

2.2 Basic Magnetic Concepts

Before the applications can be discussed, it is important to have an understanding of the basic concepts underlying the desirable properties of magnetic nanoparticles. We turn to a review by Pankhurst et al. [12] summarizing magnetic properties as they related to magnetic nanoparticles. A magnetic material placed in an external magnetic field, $H$ (units: A/m), experiences a magnetic induction of $B$ (units: T) characterized by:

$$B = \mu_0 (H + M)$$

(1)

where $\mu_0$ is the permeability of free space and $M$ is the magnetic moment:

$$M = m/V$$

(2)

where $V$ is the volume of the material and $m$ is the magnetic moment on the volume. Furthermore, all materials have some magnetic capabilities depending on the temperature and structure of their atoms. This can be represented by magnetic susceptibility, $\chi$, where

$$M = \chi H.$$ 

(3)

The above equation describes the magnetism that is induced in a material by the magnetic field, $H$. $\chi$ is a dimensionless quantity, but both $M$ and $H$ are expressed in A/m.

Paramagnetic or diamagnetic materials display little magnetic ability, and even then only under an applied magnetic field. These materials typically have magnetic
susceptibilities in the range of $10^{-6}$ to $10^{-1}$ and $-10^{-6}$ to $-10^{-3}$, respectively [3]. Materials that display ordered magnetic states even in the absence of a magnetic field are known as ferromagnets, ferrimagnets, and antiferromagnets, depending on the interaction of their electrons [13, 14]. For magnetic materials, the magnetic susceptibility depends on both the magnetic field and the temperature. Due to the saturation of $M$ at large values of $H$, $M-H$ curves are characterized by a sigmoidal curve. Specifically for ferromagnetic and ferromagnetic materials, there is an open $M-H$ curve, called a hysteresis loop, due to the pinning of magnetic domain walls when they encounter impurities such as grain boundaries. Really small magnetic particles, on the order of tens of nanometers, are single magnetic domains. This gives the particle as a whole the ability to change in response to a field while the individual particles retain their ordered state relative to each other. This unique behavior is known as superparamagnetism.

It is important to realize that a stable magnetic field will exert torque but no force on a particle; therefore, a magnetic field gradient is required to manipulate a magnetic particle. The derivation [13, 14] for force starts with the definition of a magnetic force acting on a magnetic dipole point:

$$F_m = (m \cdot \nabla) B$$

which can be expanded to:

$$F_m = V_m \Delta \chi \nabla \left( \frac{1}{2} B \cdot H \right)$$

This equation is the basis for applications such as magnetic separation and targeted magnetic drug delivery that will be discussed in the following sections.
For nanoparticles used in biological applications, the importance of optimal size cannot be underestimated. The particles must be small enough to retain their superparamagnetism. Most materials keep this property if the particle is about 5-20 nm in diameter [5]; however, nanoparticles for biologic applications must also be large enough to avoid rapid renal clearance by the body, which occurs for particles less than 10 nm in diameter [15]. Furthermore, nanoparticles with diameters greater than 30 nm are more easily filtered by the liver and spleen [16]. Taking all of the considerations into account, the working range for most biomedical applications is from 20-50 nm.

2.3 Magnetic Nanoparticle Toxicity

Magnetic nanoparticles can be made from a variety of metals including cobalt, nickel, or iron oxides. Nickel and cobalt are highly magnetic; however, they are also easily oxidized and toxic [17]. As such, biomedical applications most commonly employee iron oxides, magnetite (Fe$_3$O$_4$) or the more oxidized maghemite (γ-Fe$_2$O$_3$) [15]. Bare iron oxide nanoparticles have been shown to result in some cell cytotoxicity; however coated iron oxide nanoparticles have also been shown to be relatively biocompatible to cells [18]. It has been demonstrated that the cells remain more than 99% viable in PEG-coated iron oxide nanoparticles of concentrations up to 1 mg/mL [19]. In contrast, uncoated nanoparticles showed a 25-50% loss of viability with concentrations of only 250 µg/mL. High concentrations of approximately 2 mg/mL have been shown to lead to high cell viability losses of about 60% [20].
It is thought that the mechanism for iron oxide nanoparticle cytotoxicity is linked to cellular uptake and production of reactive oxygen species. One study demonstrated that P(PEGMA)-immobilized nanoparticles were fairly nontoxic to cells with a 93% cell viability [21]. Initially, the particles had a cell viability of 70% over the first two days of the study, which increased to 90% by the 5th day. This led the researchers to suggest that the increase in cell viability results from a decrease in the nanoparticle concentration that occurs after continued cell uptake of nanoparticles and cell mitosis. This theory follows their results that the uptake of nanoparticles decreased from 154pg/cell on the first day to 58pg/cell after 5 days [3]. Additional evidence for this theory comes the 93% cell viability study, which showed 2pg/cell update giving further support for the belief that lower cell cytotoxicity is achieved with reduced cellular uptake [22]. In one in vitro study, several metal oxide nanoparticles were studied at varying concentrations. It was demonstrated that nanoparticles are non-cytotoxic at concentrations below 100µg/mL [23, 24]. This result has been replicated by several reports identifying cytotoxic effects when nanoparticle concentrations exceed 100µg/mL.

The number of in vivo human studies are few; however, one study with dextran coated iron oxide nanoparticles induced only mild side affects as the iron oxide nanoparticles are able to be degraded and cleared from circulation with normal iron metabolic pathways [23, 24]. Ability to remove the nanoparticles is greatly impeded when nanoparticles agglomerate or which can occur for several reasons. The nanoparticles can interact with each other precipitating out of solution or they can interact
with proteins also precipitating out of solution. In general, the prevention of agglomeration, often through a polymer coating greatly improves biocompatibility [3].

2.4 Magnetic Nanoparticles in Magnetic Separation

In biomedicine, it is often necessary to separate cells of molecules out of a solution. A two step process, shown in Figure 2.1, outlines one solution using magnetic nanoparticles: 1) tagging the entity of interested with a magnetic body 2) use of a magnetic separate device to pull selectively pick the tagged entity [3]. Tagging of the magnetic nanoparticle first requires a biocompatible nanoparticle coating. Dextran, polyvinyl alcohol (PVA), polyethylene glycol, and dextran are a few options [25]. In addition to stabilization, these polymers provide sites for the attachment of targeting molecules such as antibodies or hormones [26]. When added to solutions containing the entity of interest, these magnetic nanoparticles will bind to the desired epitope. A magnetic field sufficiently strong to immobilize the particle will allow the target to be separated from the solution. It should be noted that the magnetic force must be great enough to overcome the hydrodynamic drag of the moving solution [28].

The ability of a particle to be manipulated by a magnetic field can be described by the magnetophoretic mobility. Magnetophoretic mobility increases with particle size; therefore, larger particles move faster through solutions. When choosing the application, the capability for manipulation will impact the optimal magnetic particle size. For example, the experimental time frame for cell manipulations is often relatively short, which could benefit from a micro sized particle [29]. Greater particle size comes as a cost
as larger particles increase the likelihood of interference with a test post-separation [30, 31]. After labeling, the process for magnetic separation can be as simple as holding a permanent magnetic to the container. This method is commonly limited due to slow aggregation rates [30, 31]. More efficient methods can be created with packing a flow column with a magnetizable matrix [3]; however, this can lead to problems as the
particles may irreversibility absorb on the matrix. One of the more efficient methods is the creation of magnetic field gradients through specific placement of magnets, such as in a quadrupolar arrangement in a flow column that creates a gradient radiating out from the center [32]. Magnetic separation has found several medical applications including the removal of tumor cells from circulation in the vascular system [33].

2.5 Magnetic Nanoparticles Induce Localized Hyperthermia

Magnetic nanoparticles subjected to oscillating magnetic fields have shown heating capacities resulting from the energy dissipation of the magnetic field reversals. This technology has been adapted to various fields including hardening of adhesives, development of thermosensitive polymers, and biomedicine [34, 35]. A biomedical application of interest is the use of magnetic nanoparticles for induction of localized hyperthermia. Seen in Figure 2.2, the treatment consists of dispersing magnetic particles throughout the tissue of interest. Applying an alternating magnetic field with sufficient strength and frequency will induce heating of the particles, which is immediately conducting into the surrounding tissue. Treatment for 30 minutes at a minimum of 42 °C, the therapeutic threshold, is enough to destroy cancerous cells [3]. Towards this outcome, magnetic nanoparticles have great appeal in that they offer a means to ensure non-cancerous tissue remains unharmed.
Difficult hyperthermia with magnetic nanoparticles first came under investigation in 1957 when Gilchrist et al. [36-38] investigated heating various samples with 20-100 nm iron oxide maghemite nanoparticles under a 1.2 MHz magnetic field. Important considerations for clinical treatment include the minimum dose for effective treatment, which is complicated due to the presence of blood flow [36-38]. Other important factors include the optimization of magnetic field. Both the frequency and magnitude of the magnetic field are limited due to adverse physiologic responses at high frequency fields,
which include cardiac stimulation, non-specific tissue heating, and stimulation of muscles. Generally it is accepted that magnetic fields can have frequencies of 0.05-1.2 MHz with strengths of 0-10 kA/m [40].

Since then 1957, there have been multiple in vivo studies, which describe methods to achieve the listed requirements and demonstrate the effectiveness of magnetic nanoparticle hypothermia [41] including a clinical trial demonstrating a medically significant proof of concept [42, 43]. In this study, 14 patients diagnosed with glioblastoma multiform, a severe type of brain cancer, were treated with a combination of radiotherapy and thermotherapy delivered with aminosilane coated iron oxide nanoparticles. Each tumor had multiple injection sites of 112 mgFe/mL magnetic nanoparticles with a 15 nm diameter. Tumors were injected with a volume ranging from 0.1-0.7 mL then exposed to 100 kHz magnetic field with a variable strength field ranging from 2.5-18 kA/m [3]. Recently, a magnetic hyperthermia system, NanoTherm® developed by MagForce Nanotechnologies (Berlin, Germany) has received approval in Europe for treatment of glioblastoma tumors [3]. Based on the recent advances, it is possible that magnetic nanoparticle localized hyperthermia may soon see widespread clinical use.

2.6 Magnetic Nanoparticles as MRI Contrast Agents

MRI has become a vital tool for clinical diagnosis. The underlying concept relies on applying an external magnetic field to align protons, such as hydrogen nuclei, in the body. Due to the extreme small size of a proton, there may be only three proton moments
aligned with the magnetic field \( (B_0 = 1 \, \text{T}) \) per million proton moments [44, 45]. MRI is able to work due to the exceptionally large number of protons in tissues [44, 45]. It had been estimated that there are \( 6.6 \times 10^{19} \) protons/mm\(^3\), effectively making \( 2 \times 10^{14} \) protons/mm\(^3\) observable by application of the one tesla magnetic field [46]. As described by Pankhurst et al. [47, 48], a proton returning to a disoriented state creates a relaxation signal that is measured by the current induced in the scanner coils with equations in the form of:

\[
m_z = m \left(1 - e^{-t/T_1}\right)
\]

\[
m_{x,y} = m \sin(\omega_0 t + \Phi)e^{-t/T_2}
\]

where \( T_1 \) and \( T_2 \) are the longitudinal and transverse relaxation times respectively and \( \Phi \) is a phase constant. The longitudinal relaxation time represents the loss of energy in the form of heat to the surroundings. The transverse relaxation is a relatively rapid process that occurs due to the magnetic interactions of nearby protons. Dephasing of the system can result from inhomogeneties in the longitudinal field and lead to the replacement of \( T_2 \) with \( T_2^* \):

\[
\frac{1}{T_2^*} = \frac{1}{T_2} + \gamma \frac{\Delta B_0}{2}
\]

where \( B_0 \) is variability in the magnetic field from the inhomogeneties of the field or local fluctuations in the system’s magnetic susceptibility [47, 48].

Magnetic contrast agents are often employed to shorten the relaxation time of the protons. The most common agents are gadolinium ion complexes; however,
superparamagnetic nanoparticles are a viable alternative that can noticeably shorten $T_2^*$ with less of an impact on $T_1$ [3]. Iron oxide nanoparticles are the most commonly used nanoparticle based contrast agent and exist as regulatory approved, commercial products such as Feridex, Endorem, and Resovist [50-52]. Cobalt nanoparticles have an advantage over iron oxide nanoparticles in that they have a higher magnetic saturation at room temperature [50-52], which may translate to a greater benefit to proton relaxation. The major benefit would be the creation of smaller sized particles without compromising MRI resolution. Particles smaller than 8nm are easily removed from the body through renal clearance [33], which would be an ideal pathway to remove the contrast agent post-MRI. Unfortunately, cobalt nanoparticles are significantly limited by the difficulties of synthesizing a water-soluble particle without oxidation of the cobalt core. Furthermore, the proven toxicity of cobalt sets major roadblocks towards its regulator approval for clinical use.

2.7 Magnetic nanoparticles in targeted drug delivery

Summarized by Pankhurst et al. [33, 53], most chemotherapeutics have a disadvantage in that they non-specific, meaning they will effect a wide variety of entities beyond their intended target leading to potential side-effects. This is due in part to the method of administration. In cancer treatment, many therapeutics are delivered intravenously, which results in a system wide distribution. Non-specific drugs will attack normal, healthy cells in addition to the target tumor cells. A similar result may happen with use of anti-inflammatory drugs to treat patients diagnosed with chronic arthritis.
With no targeted mechanism, high systemic doses of drug are required to achieve the needed concentration at the inflamed joint, and many patients are forced off of anti-inflammatory medications due side effects from the high dosages.

In the late 1970’s, researchers proposed targeting sites of interest using magnetic carriers controlled by an external magnetic field. The initial studies proposed using targeted magnetic drug delivery for cancerous applications [33, 53]; however, other uses have since been proposed. The ability to selectively deliver a drug of interest to a specific target is applicable to every drug that has an intended effect with an unintended consequence for non-specific action. Targeted magnetic delivery begins with the coupling of the desired therapeutic to a magnetic carrier, which can be injected into the vascular system. Placement of strong magnets around the site of interest pulls the circulating carrier to the target for delivery [55], illustrated by Figure 2.3. If the therapeutic needs to be decoupled from the carrier, multiple release mechanisms have

Figure 2.3 Targeted delivery of magnetic nanoparticles to a vascular wall. A) depiction of strong electromagnetics placed outside the body B) Close up view of directing of magnetic nanoparticles to the walls of an artery. Created by Chorney et al. [1][2][6-8][1][1] and reproduced with permission.
been suggested including enzymatic activity specific to the target or physiologic conditions such a temperature or pH [33, 56-58].

Similar to magnetic separation, the effective application requires a magnetic force generated by a magnetic field, and efficiency of delivery depends upon the strength of magnetic field and magnetic properties of the particles. Additionally, one must consider biologic factors such as circulation time, blood flow rate, and biocompatibility of the magnetic carrier. Other important parameters include tissue depth as a stronger magnetic field gradient would be required to capture particles at a longer distance. Theoretical calculations of the parameters needed to successfully produce this type of therapy have indicated that there should be a magnetic field gradient around 8 T/m for the femoral artery and 100 T/m for the carotid artery. There have been hypothesized models that may reduce some of these hurdles. Most notably the use of magnetic needles and meshes has modeled magnetic fields and gradients capable of capturing a magnetic nanoparticle in circulation.

Magnetic nanoparticles have been tested to delivery a variety of drugs both in vitro [1] and in vivo [1]. These results indicate the viability of targeted magnetic drug delivery. The field still has hurdles such as optimal dosing to overcome before clinical trials can be conducted; however, there remains great potential for this treatment mechanism.
2.8 Conclusions

Biocompatibility studies of magnetic nanoparticles have demonstrated non-toxic synthesis methods and polymer coatings opening the door to multiple applications. Their use has the potential to solve major problems in the medicine such as complications resulting from systemic delivery of drugs. As such, the use of nanoparticles has seen a huge increase over the past decade and will likely continue to grow as more applications move from bench research to clinical trials.

2.9 References


3.1 Introduction

Endovascular surgery revolutionized the treatment of arterial disease with the invention of balloon angioplasty. Since its first use in 1977, the primary limitation of the procedure has been high rates of restenosis [5]. Restenosis is the narrowing of a vessel following surgery to free up blood flow and occurs in a two part process: early thrombosis formation and long stage myointimal proliferation [6]. Following injury to the vascular wall, there is platelet aggregation to form a clot that obstructs blood flow. Fortunately, this dissipates after a few days through normal means; however, it has been proposed that the site of thrombosis decides the initiating location of myointimal proliferation [7]. The major cause of restenosis results from the proliferation of vascular smooth muscle cells (VSMCs) in a process known as neointimal hyperplasia [2].

The fate of VSMCs is of great interest to understanding the underlying mechanism of neointimal hyperplasia. Described by Melewicz et al. [8], lesions occurring in the vascular are characterized by endothelial dysfunction. This allows greater permeability of the endothelial cell barrier and leads to an accumulation of lipids and oxidation products along the arterial wall. Intimal cells respond with increased expression of adhesion proteins to recruit inflammatory cells to the site for removal of the lipids. Attached monocytes become differentiated macrophages and take up oxidized lipids leading to the formation of foam cells. The inflammatory response consisting of
macrophage recruitment and the production of cytokines, reactive oxygen species leads, and mitotic factors leads to other downstream effects. These factors induce the chemotaxis of VSMCs from the tunica media into the tunica intima to remodel the damaged vessel wall. In this review of literature, we will discuss the different phenotypes of VSMCs. Understanding the molecular cues that trigger VSMC migration and proliferation is critical to the development of treatment for neointimal hyperplasia. We will also address treatments including systemic delivery of pharmaceuticals and drug-eluting stents.

3.2 Vascular Smooth Muscle Cells

Prior to delving into the pathology of neointimal hyperplasia, we must have a fundamental understanding the underlying anatomy and physiology of the vascular system and VSMCs. In their book, McKinley and O’Loughlin [2] give an excellent introduction to of the underlying environment during normal function. The vascular system is made up of a large network of interconnected blood vessels whose function is to delivery nutrients and remove wastes. The vessels can be broken up into three groups based on the physiologic structure: arteries, veins, and capillaries. Arties and veins can be further broken down into three layers/tunics: intima, media, and externa.

The tunica intima is composed of a single layer of squamous endothelial cells, with a subendothelial lining of connective tissue. The main function of the tunica intima is to create a smooth, non-thrombogenic surface over which blood can flow. The tunica media is mainly made up of VSMCs but also contains a significant amount of
extracellular matrix such as collagen (types I, III, and V), elastin, and proteoglycans.

Through VSMCs contraction and relaxation, the tunic media can modulate vessel diameter with the goal of maintenance of steady blood flow. The outermost layer, tunica externa, is mainly composed of a protein matrix used to anchor blood vessels to surrounding tissue. The three tunics can be found in all arteries and veins; however, there will be slight differences depending on physiological location.

VSMCs have a unique ability to alter their phenotype after the developmental period of growth is over, such as in response to vascular injury leading to remodeling of
the damaged vessel [9]. Under normal conditions, VSMCs remain in their quiescent, non-
proliferative phenotype. This is often called their contractile phenotype due to the
expression of myofilaments containing contractile proteins such as α smooth muscle
actin, β myosin heavy chain, calponin, and smoothelin [10]. These filaments are aligned
into contractile apparatuses giving the VSMC a spindle shape. In response to injury,
VSMCs reduce the expression of contractile protein genes; scientists often use this gene
expression to determine the differentiated state of VSMCs [2]. In the dedifferentiated state, VSMCs proliferate, secrete enzymes to digest the extracellular matrix, and synthesize a new extracellular matrix all in an attempt to repair the injured tissue. VSMCs portraying these characteristics are termed to be in the synthetic phenotype [11, 12]. The differences in the VSMC phenotype are summarized in Figure 3.1.

![Figure 3.3 Signaling pathways that promote VSMC phenotypic switching. Abbreviations: AII indicates angiotensin II; PDGFR, PDGF receptor; Inflamm, inflammation; BMP, bone morphogenetic protein; BMPRII, bone morphogenetic protein receptor II; TK, tyrosine kinase; HDAC, histone deacetylase; Ub, ubiquitin; TCF, ternary complex factor. Original work by Mack 2011 [2]. Reproduced by permission from Wolters Kluwer Health, Inc.](image)

Much work has been done to determine what are the underlying molecular signals that could be used to initiate or maintain the synthetic phenotype. What is known about the pathways that induce VSMC differentiation can be seen in Figure 3.2. Pathways that
promote VSMC phenotypic switching can be seen in Figure 3.3. Understanding the molecular mechanisms of VSMCs is crucial to one day be able to induce a desired VSMC phenotype on command.

3.3 Treatments for Neointimal Hyperplasia

One of the first prospects to treat neointimal hyperplasia was systemic delivery of drugs. Very closely following the invention of balloon angioplasty and the subsequent identification of restenosis complications, many compounds were evaluated to treat this disease. Unfortunately, most of the drugs that have been investigated are only effective at narrow dosing ranges and have poor toleration when administered systemically [11, 12]. The next developmental phase focused on the invention of local drug delivery system allowing a high dose of therapeutic to be delivered to the artery of interest without exposing the entire body to the adverse effects. These delivery systems were found to be able achieve drug concentrations 400 to 1000 times greater than a non-specific systemic administration of the same compound [13-16]. Currently, there exist four main options under investigation for the prevention of neointimal hyperplasia: drug eluting stents, duel drug eluting stents, drug eluting balloons, and porous balloons [3]. The major advantages and disadvantages of each group are listed in Table 3.1.

The U.S. Food and Drug Administration approved drug-eluting stents in 2003 for treatment of coronary artery disease [17]. Over short term time periods, DESs show a significant reduction in neointimal hyperplasia compared to bare-metal stents [3]. There have been a variety of clinical trials testing different therapeutics with DESs including
sirolimus, everolimus, and paclitaxel [3]. While they have been readily received adaptation into clinical use, there remain a few major hurdles to overcome. Notably, there is the matter of limited drug delivery due to the small surface area and poor outcomes in peripheral arteries.[18]

Other areas of research include bioresorbable stents, which are designed to provide temporary support and restoration of vessel wall. With time, the entire stent will dissolve leaving only fully healed native tissue. Several major advantages to bioresorbable stents include ability of vasculature to maintain native tone [3], the freedom to preform later surgical revisions [18], use in pediatric patients [19], and use in patients with metal allergies [3]. Metal alloy bioresorbable stents, particularly magnesium, have advantages over polymers due to the increased strength, more rapid degradation, and minimal inflammatory responses [20]. Magnesium is the material of choice due to a 4-month degradation time and the capacity for incorporation in the body’s natural mineral supply. The evaluation of bioresorbable stents is currently in progress and in need of a large randomized clinical trial to access efficiency over DESs.

3.4 Heparin as a Drug

Heparin is a naturally produced glycosaminoglycan (GAG) that is synthesized by mast cells in connective tissue and stored in cytoplasmic secretory vesicles [21]. Heparin was discovered nearly 100 years ago and should not be confused with heparin sulfate. Discovered as an impurity in heparin, heparin sulfate is synthesized and expressed by nearly all mammalian cells and has a variety of biological functions [20]. Both heparin
and heparin sulfate and constructed from the same repeating 1-4 linked monosaccharide blocks of uronic acid and D-glucosamine. There are 24 common variations of the uronic acid/glucosamine disaccharide that comes from differences in the functional groups on the monosaccharide building blocks. The defining difference between heparin and heparin sulfate is in the proportion of disaccharides [22].

Since heparin’s initial use as an anticoagulant in 1935, it has become one of the most used natural therapeutics, second only to insulin [23]. Heparin inhibits thrombin and activated coagulation factors IX, X, XI, and XII [21, 22]. In addition, heparin inhibits proliferation of VSMCs independently of its anticoagulation effects [21, 22]. It was shown that treatment with heparin leads to increase expression of differentiated VSMC markers such as α-smooth muscle actin and calponin [21, 22, 25, 26]. Animal studies in rats demonstrated that sustained systemic injections of heparin can lead to 40-50% reduction in neointimal thickening [21, 22, 25, 26]. However, the systemic delivery of heparin in humans was reported to result increased rates of internal bleeding [23, 28]. An alternative approach is through heparin-coated stents, which have shown encouraging results supporting the use of localized delivery of heparin [23, 28]. As both an anticoagulant and VSMC proliferation inhibitor, heparin remains a drug of interest in the prevention of neointimal hyperplasia.

3.5 Conclusions

Neointimal hyperplasia remains one of the major complications associated with endovascular and vascular surgery. The underlying cause is the proliferation of VSMCs,
which in response to vascular injury undergo a phenotypic shift to repair the integrity of the wall. In the past few decades, there has been significant progress towards preventing this disease with the widespread clinical use of drug-eluting stents; however, there still remain unsolved problems. New prospects, such as the localized delivery of natural proliferation inhibitors, may be able to improve clinical outcomes.
Table 3.1 Summary of common investigated options for localized drug delivery

Original work by Seedial et al. [6-8] Reproduced with permissions from Elsevier

<table>
<thead>
<tr>
<th>Intraluminal devices</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Current status</th>
</tr>
</thead>
</table>
| Drug-eluting stent   | • Reduced incidence of restenosis  
                      • Effectively prevents restenosis in coronary vessels  
                      • Safe  
                      • Lack of systemic toxicity  
                      • Prevents sub-acute recoil | • Poor results in peripheral arteries  
                      • Hypersensitivity reactions  
                      • Costly  
                      • Incomplete reendothelialization  
                      • Late stent thrombosis  
                      • Lifelong antiplatelet therapy  
                      • Localized aneurysms  
                      • Drug delivery not uniform  
                      • Limited drug delivery due to small surface area of stent | Currently in clinical use in the coronary circulation |
| Dual drug-eluting stent | • Can release both antiproliferative and antithrombotic drugs (at different rates)  
                          • Reduction in restenosis  
                          • Antirestenotic efficacy seen at long-term follow-up at 2 years | • Same as single-drug stents | Currently in clinical trials |
| Drug-eluting balloon | • Homogeneous drug delivery to vessel wall  
                        • Limited need for antiplatelet therapy  
                        • No stent thrombosis  
                        • No stent scaffolding to disrupt patterns of flow  
                        • Can be used in very small vessels  
                        • Immediate drug release  
                        • Simultaneous plaque compression and drug delivery  
                        • No residual foreign body | • Not as effective as DES in coronary vessels  
                        • Elastic recoil  
                        • Negative remodeling  
                        • Cost  
                        • One-time use | Currently in clinical trials |
| Porous balloon       | • More effective drug delivery through a balloon | • Vascular barotrauma due to jetting  
                        • Holes can become obstructed leading to nonhomogeneous drug delivery  
                        • Systemic release of drug | Currently in clinical trials |
3.6 References


CHAPTER FOUR

SYNTHESIS AND CHARACTERIZATION OF HEPARIN COATED MAGNETIC NANOPARTICLES

4.1 Abstract

Purpose:

Magnetic nanoparticles have potential for use in a wide variety of fields. One advantage to magnetic nanoparticles is their controllability with an external magnetic field making them an excellent choice for targeted drug delivery. Heparin is the drug of interest for this study and has long been known to inhibit proliferation of vascular cells. It has been suggested for use in treating neointimal hyperplasia to slow vascular smooth muscle cell growth. Localized heparin delivery approaches have shown promise. Therefore, the purpose of this study is to synthesize and characterize a heparin coated magnetic nanoparticle for targeted delivery.

Materials and Methods: Magnetite nanoparticles were created using thermal decomposition of iron acetylacetonate and oleic acid. Poly (ethylene glycol) was used for steric hindrance stabilization. Nanoparticles were characterized using TEM, FTIR, hydrodynamic diameter, zeta potential and DMMB assays.

Results: A uniform distribution of nanoparticles was synthesized with a diameter of 24.3 +1.9 nm. Analysis of DLS results indicated that the PEG polymer successfully replaced the oleic acid surfactant. Variability among nanoparticle batches led to a heparin loading from 0.1056 to 3.1 gram of heparin per gram of nanoparticle.
**Conclusions:** These results indicate the successful synthesis of heparin-coated nanoparticles; however, variability among batches suggests that the process requires further investigation for refinement.

### 4.2 Introduction

Magnetic nanoparticles have great interest for researchers due to their potential for use in a wide variety of applications due to their responsiveness to a magnetic field. They have already seen use in many disciplines including the creation of magnetic fluids, data storage, and biomedicine [2]. In particular superparamagnetic nanoparticles have high potential for multiple biomedical applications including hyperthermia, magnetic resonance imaging, tissue repair, targeted drug delivery, and cellular therapy [3, 4]. Most applications require the size of the nanoparticle remain below a critical value, typically around 20nm [5]. One major hurdle in the development of nanoparticles is controlled synthesis to achieve this optimal particle size.

There exist multiple methods to synthesize magnetic nanoparticles. Depending on the desired outcome, researchers may choose an efficient method to produce highly stable, shape-controlled and monodisperse magnetic nanoparticles. Popular methods are hydrothermal synthesis, microemulsion, co-precipitation, and thermal decomposition [2]. Thermal decomposition was inspired by the synthesis of high-resolution nanocrystalline semiconductors [6] and is of particular appeal for biomedical applications due to the ability to achieve nanoparticles with a monodisperse size and shape. Typical synthesis occurs with the thermal decomposition of organometallic precursors such as metal acetylacetoneates [7] in organic solvents with high boiling points such as fatty acids [8]
or hexadecylamine [9]. Changing the starting ratios of the organometallic compound and surfactant controls nanoparticle size and shape. Fine tuning of the these properties is achieved by controlling the reaction time and reaction temperature [2]. Using the thermal decomposition method, monodisperse Fe₃O₄ nanocrystals with diameters ranging from 3-50 nm have been created demonstrating the effectiveness and versatility of the method [10]. There exist several material choices for the synthesis of magnetic nanoparticles including iron, cobalt, and nickel [2]. For biomedical applications, iron oxides, such as magnetite or maghemite, have proven to be superior to alternative options due to increased biocompatibility and stability [5, 11-13]. From here on, we will refer to iron oxide magnetic nanoparticles when discussing magnetic nanoparticles.

The importance of narrow size distribution is of critical importance to the functionality of the magnetic nanoparticle. The ideal range for magnetite is around 5-20 nm [5]. At this size, each nanoparticle behaves as a single magnetic domain displaying superparamagnetic behavior. Smaller particles have greater tendency to not agglomerate due to their smaller magnetic moments, so the magnetic force between particles is reduced. Typical stabilization methods can be broken down into two major groups: electrostatic repulsion or steric hindrance. There exist multiple steric strategies that all end up with a similar outcome, a magnetic nanoparticle core inside of a protected by a shell structure that physically blocks neighboring particles from sticking together. The common mechanisms for protection include coating with surfactants[14], polymers[15, 16], silica [17], carbon [18], or precious metals [19]. Some of most investigated polymer coatings are dextran, starch, chitosen, polyethylene glycol (PEG), and polyvinyl alcohol (PVA) [20]. Besides being used for steric hinderance, some of these polymers can be
used for attachment of additional targeting ligands for biological targeting. In general, for biological applications, hydrophilic polymer coatings have a significant advantage providing greater biocompatibility [21] and allowing the option for additional modifications to add functionality.

One such possibility for added functionality is the biological molecule, heparin. Heparin is a small linear glycosaminoglycan that is commonly used as an anti-coagulant [22]. In addition to anti-coagulation, the medical community has known for decades of the anti-proliferative effects of heparin [23, 24]. Early clinical trials of systemic heparin administration to prevent neointimal hyperplasia proved to be ineffective [25] with an increase in minor bleeding [26]. However, localized delivery of heparin has been shown to be effective at decreasing vascular smooth cell proliferation without increased internal bleeding risk [27]. Biocompatible magnetic nanoparticles conjugated with heparin could provide a novel targeted heparin therapy; magnetic fields could be used to keep the heparin particles localized to the sites of interest. Here, we describe the synthesis and characterization of heparin-coated iron oxide magnetite nanoparticles using the thermal decomposition method for the creation of uniform particle shape and size.
4.3 Materials and Methods

Synthesis

Magnetite nanoparticles were synthesized using the a modified version of Sun’s method [28]. For this study, iron (III) acetylacetonate (Fe(acac)$_3$) (Fluka) was used as the iron precursor and oleic acid was employed as the ligand. Using previous work to aim for a target nanoparticle diameter of 25 nm, we combined Fe(acac)$_3$ with oleic acid (Alfa Aesar) in a 1:15 molar ratio. As an example, 1.078g (3 mmol) of Fe(acac)$_3$ was combined with oleic acid in a 1:15 molar ratio.
with 13.48g (47.6 mmol) of oleic acid in a three-neck round bottom flask. The reaction took place under flowing nitrogen at 0.1 liter per minute while stirring with an overhead stir bar. The solution was heated up to the refluxing temperature of 360 °C and held there for one hour.

The reaction solution was then allowed to cool to room temperature. The resulting product was suspended in hexanes to transfer to centrifuge tubes and was washed three times by precipitation with acetone followed by resuspension with hexanes. After the final wash, the nanoparticles were left in a drying oven for two days to remove remaining traces of solvent. The dried particles were suspended in tetrahydrofuran (THF) (Fisher Scientific) and further purified using THF gel permeation chromatography (GPC) of Bio Beads S-X1 Support (Bio Rad 152-2151) packed in a glass column (40 cm long, 2.5 cm diameter) and pressurized with 5 psi N₂ gas. Nanoparticles were either immediately used in a ligand exchange or kept in THF for long-term storage.

![Figure 4.2 PEG polymer used in ligand exchange to stabilize the nanoparticle and improve biocompatibility](image-url)
Ligand Exchange

Use of nanoparticles in biological applications requires hydrophilicity; therefore, the previously described hydrophobic particles were modified with a hydrophilic ligand. Previously, our lab has synthesized and characterized a hydrophilic polymer consisting of nitroDOPA anchors attached to 1,800 g/mol poly (acrylic acid) (Sigma-Aldrich) with a 8,000 g/mol poly (ethylene glycol) (Sigma-Aldrich) spacer terminated in an alcohol, seen in Figure 4.2. We used excess polymer (250mg) dissolving it in 5 mL chloroform (Fisher Scientific) and back filling with inert nitrogen gas. From the particle synthesis, we removed the THF solvent by rotary evaporation. The product was suspended in 5mL of chloroform and added drop-wise to the polymer solution over 15 minutes while under sonication. The resulting reaction was placed on a shake plate and agitated for 7 days. To remove unreacted polymer, the post-reaction product was run through a THF GPC.

Modification with Heparin

In order to attach heparin, polymer modification was required to change the terminal alcohol to an amine group. The reaction was performed under a nitrogen gas blanket. Excess 3-chloropropylamine (Sigma) was dissolved in 5 mL of dichloromethane (DCM) (Acros Organics). Triethylamine (Fisher Scientific), utilized as a scavenger for acid, was combined with the 3-chloropropylamine in molar ratio 1:3. The nanoparticle mixture was added drop-wise into the reaction. The reaction was placed on a shake plate and agitated for 2 days.
Carboxylic acid groups of heparin were covalently bonded to the primary amine groups on the nanoparticle ligand using standard EDC/NHS chemistry. The concentration of nanoparticle solution was determined with an iron determination assay leading to the calculation of the number of free ligands in solution. Molar ratios of 1 ligand : 3 heparin (MW 3000g/mol, MP Biomedicals) : 30 EDC (TGI) : 65 sulfo-NHS (Thermo Scientific) were dissolved in deionized water. Heparin, EDC, and sulfo-NHS were combined and allowed to equilibrate for 15 minutes before adding the nanoparticle mixture. The reaction was allowed to proceed for 12 hours at room temperature. Heparin coated nanoparticles were purified by running through a water column. The proposed synthesized nanoparticle can be seen in Figure 4.3.

![Key]

- Fe₃O₄ Core
- nitroDOPA
- Poly (acylic acid)
- Poly (ethylene glycol)
- Propylamine
- Heparin

Figure 4.3 Proposed synthesized heparin nanoparticle
Characterization

Following the initial synthesis reaction, transmission electron microscopy (TEM) was used to measure the size distribution of the nanoparticle solution. Diluted (~0.1mg Fe₃O₄ per mL) hexane solution was dropped onto a copper grid coated with a carbon film. Using a Hitachi H-9500 instrument with an accelerating voltage of 300 kV, high resolution images were obtained at 200,000X and 600,000X. Using the Feret’s diameter function from ImageJ’s particle analysis, approximately 300 nanoparticles were measured to determine the size distribution. Before and after the addition of the terminal amine, Fourier transform infrared spectra (FTIR) was taken by Thermo-Nicolet Magna 550 FTIR to confirm the presence of a primary amine with peaks of 1580-1650 cm⁻¹ and 3320-3520 cm⁻¹.

At each step in the synthesis of heparin coated nanoparticles, dynamic light scattering (DLS) was used to estimate the hydrodynamic diameter. Measurements were made in dH₂O with a Malvern Zetasizer NanoZS. We report the intensity-weighted sizes. Zeta potential is defined to be the electric potential at a small distance from the surface of the particle (i.e. the slipping plane) relative to a point far away in solution. For simplification, it can be thought of as the potential difference between the electrostatically bound fluid and the dispersive fluid. As an important parameter to understand the colloidal stability through electrostatic charges, zeta potential at the various stages was carried out also using capillary cell electrophoresis technique and the Zetasizer NanoZS.
Iron Determination Assay

A modified previously described method [29] was used to find the concentration of iron in solution. For the full descriptive protocol, see Appendix A. In short, working solutions of 100 g/L of hydroxylamine hydrochloride (Sigma-Aldrich) in deionized water (dH₂O), 3 g/L of 1,10-phenanthroline (Sigma-Aldrich) in 10% methanol (Honeywell Burdick and Jackson) diluted with dH₂O, and 100 g/L sodium acetate (VWR) in 20% glacial acetic (Mallinckrock Chemicals) acid diluted with dH₂O. A standard curve was created from the dissolved iron and developed by successive additions of hydroxylamine hydrochloride, 1,10-phenanthroline, and sodium acetate-acetic acid. Background absorbance, read at 690 nm, were subtracted from sample absorbance read 511nm with a

Figure 4.4. Schematic of ions away from a particle and resulting electrical potential as a function of distance from the particle surface. Taken from http://en.wikipedia.org/wiki/Zeta_potential and reproduced in accordance to CC BY-SA 3.0 [5]
UV-Vis spectrophotometer. Using the nanoparticle diameter from TEM analysis, density (5.2 g/cm$^3$) of magnetite [30], and volume of solution, the concentration of nanoparticle solution was calculated

Dimethyl Methylene Blue Assay

Glycosaminoglycans are long unbranched polysaccharides that are made up of a repeating disaccharide unit consisting of a hexosamine (glucosamine or galactosamine) and a hexuronic acid (glucuronic or iduronic acid). A previously described method [31, 32] describes the method for quantification of sulfated GAG content using the colorimetric indicator, 1,9-dimethylmethylene (DMMB) (Sigma), which undergoes a shift in its absorption spectrum when bound to sulfated GAGs. DMMB assays were used to quantify the amount of heparin attached on the nanoparticles and confirm the success of heparin attachment.

Using a round-bottom 96 well plate, 180µL of DMMB solution was added to 20µL of sample. Standard curves 0-100µg/mL of heparin and chondroitin sulfate (Sigma) were found to not be significantly different (see Appendix C). Samples were agitated for 5 seconds, and the absorbance at 530nm was immediately read using a microplate reader. The coating of heparin/nanoparticle was determined using the measured DMMB absorbance, the linear regression equation, and the concentration of nanoparticle from the iron determination assay.
4.4 Results

Analyzing the TEM images (Figure 4.5), it is evident that the synthesized nanoparticles form a uniform distribution. The average diameter was found to be $24.3 \pm 1.9$ nm. This value was significantly lower than the dynamic light scattering estimated hydrodynamic diameter of 64.43 nm. Following the ligand exchange of oleic acid for nitroDOPA-PAA-PEO-OH the hydrodynamic diameter was notability increased to 150.1 nm. Ensuring polymer modifications led to slight hydrodynamic diameter decreases first to 149.1 nm following the 3-chloropropylamine reaction replacing the terminal alcohol with a primary amine group. After the EDC/NHS reaction attaching heparin to the nanoparticle, we saw a dip down to a hydrodynamic diameter of 131.6 nm.

Figure 4.5 TEM Results a) TEM image of uncoated iron oxide particles. b) Histogram of nanoparticle magnetic core diameter
Figure 4.6. FTIR spectrum after addition of terminal amine group to polymer

Figure 4.7 Dynamic light scattering hydrodynamic diameters of particles
After replacement of the oleic acid ligand with the PEO polymer, the reaction was water-soluble and zeta potential measurements were taken at subsequent steps. The zeta potential of the PEGylated nanoparticles was found to be -0.809 mV. Addition of the 3-chloropropylamine led to an increase up to a zeta potential of 1.07 mV. Following the EDC/NHS chemistry, the zeta potential dropped to -3.74 mV.

After the EDC/NHS attachment of heparin, the product was purified by running it through a water column. The output was collected in 5mL fractions. Fractions that contained nanoparticles were determined by the presence of a brownish tint color seen in fractions three to nine of Figure 4.8. Positive DMMB assays of tinted fractions confirmed both the success of heparin nanoparticle synthesis. The purification profile can be seen in Figure 4.8 showing the concentration of heparin determined by a DMMB assay. The distribution shows a distinctive bimodal shape indicating both the attachment of heparin and the purification of the nanoparticle were successful.

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Hydrodynamic Diameter/Z-Avg. (nm)</th>
<th>Zeta-Potential (mV)</th>
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</thead>
<tbody>
<tr>
<td>Fe₃O₄ (NP)</td>
<td>64.43</td>
<td>-</td>
</tr>
<tr>
<td>NP-OH</td>
<td>150.1</td>
<td>-0.809</td>
</tr>
<tr>
<td>NP-NH₂</td>
<td>149.1</td>
<td>1.07</td>
</tr>
<tr>
<td>NP-Hep</td>
<td>131.6</td>
<td>-3.74</td>
</tr>
</tbody>
</table>

Table 4.1 Summary of hydrodynamic diameter and zeta potential
The theoretical loading of heparin on a nanoparticle was calculated using the diameter from TEM analysis and the assumption of 5 free ligands/nm$^2$. This value was determined to be approximately 1.2 grams of heparin per gram of nanoparticle (approximately 9400 heparin molecules/nanoparticle). The actual loading heparin was determined using a combination of an iron determination assay and DMMB assay. Shown in Figure 4.8, three concentrations of nanoparticles were measured using the DMMB assay and fitted with linear regression to return the slope value of 2.889 heparin/µg nanoparticle (approximately 22,500 heparin molecules/nanoparticle). We also show the lack of GAGs on the control nanoparticle. The heparin loading was repeated on
a later batch showing only 0.976 µg heparin/µg nanoparticle (approximately 7,600 heparin molecules/nanoparticle).

![Graph](image)

Figure 4.9 Determination of heparin loading on nanoparticles by iron assay and DMMB assays

4.5 Discussion

Magnetic nanoparticles have a wide variety of applications; however, the properties depend upon the size meaning that a narrow size and shape distribution is of utmost importance for tailoring to specific applications [33]. For most envisioned applications, researchers typically desire to have nanoparticle diameters in the 10-50nm range [2]. In our synthesis, our goal for optimal nanoparticle range was 20-30 nm for increased circulation time. Particles must be larger than 10 nm to avoid rapid removal by the kidneys [34, 35]. However of the nanoparticles large enough to avoid renal clearance, it has been shown that larger particles are easier to remove [36, 37]. In addition, larger
size particles lose the capacity for superparamagnetic behavior [2], leaving the ideal size for targeted heparin delivery to be in the 20-30 nm range. As seen in 4.3, it is evident that we have achieved a uniform distribution directly in the center of our target range.

Due to the use of oleic acid as the surfactant for the synthesis, nanoparticles immediately resulting from the reaction are hydrophobic. Biological use requires the solubility in aqueous solutions justifying our ligand exchange. As a biocompatible hydrophilic polymer, PEG coatings have been seen widespread use through the medical field in a variety of applications [21]. In addition to improve biocompatibility, the use of a PEG brush provides stabilization to our particles prevent agglomeration through steric hindrance. Multiple studies have sought to take advantage of this double benefit [38, 39].

As part of a verification step, FTIR was used to confirm the presence of primary amines following the modification step of the PEG polymer with 3-chloropropylamine. It is known that primary amines display a small to medium peak with a wavenumber in the range of 1580-1650 cm\(^{-1}\) and also at 3320-3520 cm\(^{-1}\) [40], marked in Figure 4.6. This result was interpreted as successful modification, and the synthesis proceeded to next step with heparin attachment.

The bonding of heparin was verified with two methods: measurement of zeta potential and DMB assay. The zeta potential was measured at the three polymer modification steps during the synthesis. Following the ligand exchange, we measured a zeta potential of -0.809 mV, which is consistent with other work showing PEG coated nanoparticles to have an approximately neutral zeta potential [41]. Following the addition of a primary amine, we noted an increase in zeta potential to 1.07 mV. This also a consistent change following modification with a primary amine [42]. Finally, heparin
modification led to a decrease in zeta potential down to -3.74 mV. As heparin has one of the highest negative charge densities of known biological molecules [43], it is unsurprising that the addition of heparin leads to a decrease in zeta potential. This was used as verification to confirm the successful modification of heparin to the nanoparticle. Studies with other heparin-coated nanoparticles have shown zeta potentials ranging from -10 to -40 mV [44, 45]. Additionally, the smaller magnitude zeta potentials of our particles is likely due to the polyethylenimine brush coating as the use of a brush coating of polyethylenimine for steric stabilization has been shown to result in particles with a more positive surface charge [44].

The combination of iron determination and DMMB assays were used to quantify the loading of heparin onto the magnetite nanoparticles. Two different batches of heparin nanoparticle synthesis underwent quantification, shown in Figure 4.9. It is evident that the process is very batch dependent with the first batch testing yielding 2.889 ug heparin / ug nanoparticle, while the second batch was loaded at 0.976 ug heparin / ug nanoparticle. This is close to a 3-fold difference in the concentration and could have potential for larger impact on the biological level. Batch differences may be due to problems that occurred when the synthesis method used to create the high loaded group was scaled up, which resulted in the low loading batch. While the ratios of the reactants were kept constant between synthesis batches, the reactant concentrations were increased by approximately 5 times in the scale up while maintaining the same volume. As an negatively charged molecule at pH values greater than 3 [46], heparin has a natural electrostatic repulsion for itself, which may have resulted in a lower loading when the reaction concentration was increased.
It is this same the localized charge density from binding so many heparin molecules in close proximity that we believe plays a major role in the cellular response that we observed in the next chapter.

4.6 References


CHAPTER FIVE
EFFECTS OF HEPARIN-COATED MAGNETIC NANOPARTICLES ON VASCULAR SMOOTH MUSCLE CELLS

5.1 Abstract

*Purpose:* Neointimal hyperplasia is one of the main problems facing endovascular surgery. Nanoparticles, which induce a vascular smooth muscle cell (VSMC) phenotypic shift towards the quiescent contractile phenotype, would have a huge impact on the treatment of patients after endovascular surgery. In pursuit of this, we have developed a heparin-coated nanoparticle. This study describes the *in vitro* testing of heparin-coated magnetite nanoparticles on VSMCs.

*Materials and Methods:* VSMCs were treated with 0-100µg/ml concentrations of heparin-coated nanoparticles. Cell toxicity was measured with MTS and Live/Dead assays. The cell morphology changes in response of the nanoparticle were characterized with Prussian Blue Stain and immunofluorescence staining of alpha smooth muscle actin fibers.

*Results:* We observed inhibition of proliferation of VSMCs in a dose dependent fashion with noticeable changes in cell number occurring at doses as low as 1 µg/mL. Observation of alpha smooth muscle actin fibers suggested possible alignment of fibers indicative of VSMC phenotype change.
Conclusions: Toxicity/proliferation studies indicated that heparin nanoparticles have potential for use in the clinic. Further investigation is required towards the optimal dosing and further characterization of VSMC markers characteristic of the contractile phenotype.

5.2 Introduction

Presently, one of the major problems facing vascular surgery is neointimal hyperplasia, caused by the excessive proliferation of vascular smooth muscle cells. During neointimal hyperplasia, VSMCs loose contractile proteins, while increasing their proliferation, production of extracellular matrix, and migration [1]. This process describes a shift from the contractile phenotype towards the synthetic/proliferative phenotype. The defining feature of the contractile phenotype is the presence of a contractile apparatus containing smooth muscle α-actin, smooth muscle myosin heavy chain, calponin, and SM-22 [1, 2]. Of these proteins, smooth muscle α-actin is the most commonly used marker of differentiated lineage.

Heparin has been shown to inhibit proliferation of VSMCs both in vitro and in vivo [3-6]. While this effect has known for some time, there still remains much unknown about the exact mechanism through which heparin takes action. It is known that heparin can be internalized [7] through cell surface heparin sulfate proteoglycans [8]. Furthermore, heparin can activate protein kinase R, which leads to blocking of the G1 to S-phase transition of the cell cycle [9]. Alternative suggested mechanisms include the interaction with an unknown surface receptor leading to activation of protein phosphatases that can modulate the cell cycle progression [10-12]. The degree of
sulfation impacts the anti-proliferative properties; however, no critical structural motif or sulfate location seems to be the responsible party for heparin’s effects [13-17].

Superparamagnetic iron oxide nanoparticles (SPIONs) are of particular interest for biomedical applications requiring a magnetized particle, due to their reduced cytotoxicity and stability compared to other magnetic nanoparticles [18-21]. Depending on the study, bare metal SPIONs exert some toxic effects when treated at concentrations ranging from 50-250 µg/mL [18]. Coating the nanoparticle with a biocompatible polymer can reduce the toxicity. It has shown that coating with poly (ethylene glycol) allowed cells to remain 99% viable at a concentration as high as 1mg/mL [22]. Overall, it is very hard to determine the viability and optimal dosing of a nanoparticle before cellular testing, partially because nanoparticle concentrations and sizes vary widely in the literature during toxicity testing. Further complicating the issue is the fact that different research groups have tested their particles on a many different cell lines.

The first step in understanding a potential treatment effect in the body is in vitro cellular studies as they allow researchers to control experimental conditions. Cells are sensitive to fluctuations in nutrients, waste concentrations, pH, and temperature [23], which allows researchers to focus in on the underlying mechanism of cellular responses. Therefore, cellular testing is ideal for testing of nanoparticle toxicity and characterization of the mechanism through which the particle exhibits its effect giving researchers validation of potential treatments before ever testing in the body.

One of the simplest means to assess cellular condition is qualitative observation through bright-field microscopy. This technique can be useful in assessing toxicity when
there is a noticeable change in cell or nuclear morphology [24]. However, the more common method is a quantitative assay that assesses cell viability through a colorimetric change. Different quantitative tests have been developed to test various cellular functions. Common tests include the Live/Dead viability test [25], lactate dehydrogenase release monitoring [26-28], and MTT/MTS viability assays [29, 30], which test for intact cellular membranes, number of damaged/dead cells, and metabolic activity respectively.

This study describes the merge of nanotechnology with the antiproliferative effects of heparin. Using the heparin-coated nanoparticles from Chapter Four, their potential for use in treatment of neointimal hyperplasia is evaluated through toxicity studies on VSMCs and characterization the mechanism of action at early time points.

5.3 Materials and Methods

Cell culture

Aortic vascular smooth muscle cells were isolated from adult Sprague Dawley rats with the protocol described in Appendix D. The rat VSMCs were cultured in High Glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin/streptomycin (HyClone). VSMCs were grown to 70-80% confluence in T-75 flasks at 37°C and 5% CO₂, replacing cell culture media every 48 hours. Cells were passaged using 0.25% trypsin with 0.02% ethylenediaminetetraacetic acid (HyClone). Cells between passage 6 and 10 were used in experiments.
Nanoparticles

Heparin coated nanoparticles (NP-Hep) were synthesized and characterized as described in Chapter Four. The amine terminated particle (NP-NH$_2$) from the last step before addition of heparin through EDC/NHS chemistry was used as a control particle throughout all testing.

MTS Assay

The proliferation of cells was measured using the CellTiter AQ$_{ueous}$ One Solution Cell Proliferation Assay kit (Promega). The detection of viable cells is based on the capacity of cells to reduce the MTS tetrazolium compound, $[3-(4,5\text{-dimethylthiazol-2-yl})$-$5-(3\text{-carboxymethoxyphenyl})$-$2-(4\text{-sulfophenyl})$-$2\text{H}$-$tetrazolium$, into a colored formazan product that is soluble in cell culture media. Cells were seeded at 2,000 cells/well into a 96-well assay plate and allowed 24 hours to adhere and grow with normal media conditions. The media was removed and replaced with nanoparticle solutions using triplicates. Nanoparticle solutions contained 1, 10, 25, 50, and 100 µg/mL of nanoparticles. Controls consisted of NP-NH$_2$ and heparin (MW≈3,000 g/mol, MP Biomedicals) at concentrations of 6.25, 25, 100, 400, 1600, and 3200 µg/mL. Cells were incubated with experimental conditions for 48 hours. Experimental media was removed, and cells were washed with PBS three times to remove nanoparticles. Assays were performed using 1:5 ratio of the CellTiter AQ$_{ueous}$ One Solution Reagent diluted in PBS. The assay was allowed to proceed for 2 hours at 37°C and 5% CO$_2$. Absorbance of
the formazan product was read at 490 nm using a microplate reader. Presented cell proliferation represents absorbance normalized to the 0ug/mL PBS control.

Live/Dead

Cells were seeded at 25,000 cells/well into a 24-well assay plate and allowed 3 days to adhere and grow with normal media conditions. The media was removed and replaced with nanoparticle solutions. Nanoparticle solutions contained 1, 10, 25, 50, and 100 µg/mL of nanoparticles. Heparin (MP Biomedicals) concentrations of 6.25, 25, 100, 400, 1600, and 3200 µg/mL were also tested to determine inhibitory effects of the native molecule. Cells were incubated with experimental conditions for 48 hours. Following experimental treatment, a negative control was created by treatment with 70% ethanol for 30 minutes at room temperature. All cells were washed three times with PBS (MP Biomedicals). Using a Live/Dead Viability/Cytotoxicity kit (Life Technologies), cells were incubated for 30 minutes with 2µM calcein acetoxyethyl and 4µM ethidium homodimer-1 diluted in PBS. Cells were imaged with fluorescent microscopy, and fluorescence was measured using a microplate reader. Presented cell viability represents fluorescence normalized to the 0ug/mL PBS control.

Prussian Blue Stain

The cellular uptake of nanoparticles was characterized with a Prussian Blue Stain kit (Polysciences). Cells were seeded at 5,000 cells/well into a 96-well assay plate and allowed 24 hours to adhere and grow with normal media conditions. The media was
removed and replaced with nanoparticle solutions using duplicates. Cells were treated with media containing 1, 10, and 100 µg/mL of nanoparticles for 2 hours. Equal amounts of 4% potassium ferrocyanide and 4% hydrochloric acid were mixed to create a working solution. Cells were covered with the working solution for 10 minutes after which the solution was replaced for another 10-minute incubation. The cells were washed with PBS before counterstaining with Nuclear Fast Red for 3 minutes. Phase contrast images were taken using an Olympus CKX41 microscope.

Immunofluorescence Staining

Immunofluorescence staining was performed to stain the smooth muscle alpha-actin to determine if there is a phenotypic change based on the alpha-actin content and fiber distribution. Cells were seeded at 2,000 cells/well into a 96-well assay plate and allowed 24 hours to adhere and grow with normal media conditions. The media was removed and replaced with nanoparticle solutions using duplicates. Nanoparticle solutions contained 1 and 10 µg/mL of nanoparticles. Cells were treated with the experimental conditions for 48 hours, then fixed with 4% paraformaldehyde (Sigma) for 10 minutes and washed 3 times with PBS. Permeabilization occurred for 15 minutes with PBS / 0.01M glycine (Acros Organics) / 0.1% Triton X-100 (Sigma) and blocked with 5% BSA/PBS followed by 5% donkey serum /1% BSA/PBS. The cells were incubated overnight at 4°C with primary antibody rabbit polyclonal to alpha smooth muscle actin (Abcam ab5694) in 1% BSA/PBS. Next, the cells were washed twice with 1% BSA/PBS followed by 5% donkey serum /1% BSA/PBS. Secondary antibody, Donkey anti-Rabbit
IgG (H+L) Secondary Antibody conjugated with Alexa Fluor® 488 (Invitrogen A21206) was diluted 1:100 in PBS and incubated with samples for 2 hours. Cells were washed with 1% BSA/PBS followed by pure PBS before staining for nuclei with DAPI at 300 nM diluted in PBS. Cells were washed a final time with PBS before fluorescent imaging with the EVOS FL Imaging System.

Statistical Analysis

Statistical analysis was performed in R Studio. MTS and Live/Dead relative viabilities were tested for the equal variance assumption to use the Student t-test. If groups failed the equal variance test, then Welch Two Sample t-test was used. For comparison of multiple groups, the assumption of equal variance was tested using the Bartlett test of homogeneity of variances and Fligner-Killeen test of homogeneity of variances. One-way analysis of variance (ANOVA) followed by Tukey’s Honestly Significant Difference (HSD) test was performed to test for statistical significance. Results were considered significant for p < 0.05.

5.4 Results

Heparin coated nanoparticles inhibited the proliferation of VSMCs in a dose dependent fashion measured by relative MTS absorbance shown in Figure 5.1. Based on the morphology (images not shown) and MTS absorbance, control amine terminated nanoparticles resulted in cell death at all concentrations (p < 0.001). Heparin nanoparticles produced a significant decrease in VSMC proliferation at 1 µg/mL (p <
0.05) with increasing inhibition as the concentration increased up to 100 µg/mL. At 100 µg/mL, the effects of heparin nanoparticles become indistinguishable from that of the amine nanoparticles. Based on the DMMB assay to determine heparin loading from Chapter Four, 1 µg of nanoparticle was coated with 0.976 µg of heparin.

![MTS relative viability of VMSCs after 48 hours of treatment with nanoparticles](image)

Figure 5.1. MTS relative viability of VMSCs after 48 hours of treatment with nanoparticles. Note: 1 µg of Fe₃O₄ is equivalent to 0.976 µg of heparin. * Denotes statistical significance relative to 0 µg/mL (p<0.05)

As a second measure of cell viability, Live/Dead was used to both quantitative and qualitatively compare with the MTS results. It is important to note that the nanoparticles used in the Live/Dead experiment are from a different batch than all other
experiments described in this paper. This batch of nanoparticles was loaded with 2.8896 µg of heparin per µg of nanoparticle. As seen in Figure 5.2, concentrations greater than 10 µg/mL of heparin nanoparticles reduced relative cell viability (p < 0.005). Amine terminated nanoparticles significantly lowered cell viability at 25 µg/mL (p < 0.005). These values are consistent with the fluorescent images seen in Figure 5.3. At 1 µg/mL for both nanoparticles, the live cells (shown in green) appear consistent in number and morphology with that of the positive control. At the critical concentration of 25 µg/mL.

Figure 5.2 Live/Dead relative viability of VMSCs after 48 hours of treatment with nanoparticles. Note: 1 µg of Fe₃O₄ is equivalent to 2.8896 µg of heparin. * Denotes statistical significance relative to 0 µg/mL (p<0.05)
and beyond, viable cells no longer exist shown by a noticeable absence of cells live or dead (shown in red) in Figure 5.3.
Figure 5.3 Fluorescent pictures of Live/Dead Stain (Live cells are shown in green and dead cells in red), 10x magnification for varying nanoparticle: a) 1 μg/mL NP-Hep b) 10 μg/mL NP-Hep c) 25 μg/mL NP-Hep d) 1 μg/mL NP-NH₂ e) 10 μg/mL NP-NH₂ f) 100 μg/mL NP-NH₂ g) positive h) negative control
Previously, experiments testing a range of concentrations of heparin (0-3200 µg/mL) have shown to inhibit proliferation of VSMCs at concentrations as low as 25 µg/mL [31]. For comparison, the same concentration range was tested with MTS and Live/Dead in this paper. Shown in Figure 5.4 and Figure 5.5, the heparin used in this experiment did not produce any significant change in VSMCs at any concentration. Observation of the fluorescent images, Figure 5.6, shows no difference in cell number or phenotype supporting the quantitative measurements of the MTS and Live/Dead assays.

Figure 5.5 MTS and Live/Dead relative viability of VMSCs after 48 hours of treatment with heparin.
Figure 5.6 Fluorescent pictures of Live/Dead Stain (Live cells are shown in green and dead cells in red), 10x magnification for varying heparin concentrations: a) 0 ug/mL b) 6.25 ug/mL c) 25 ug/mL d) 100 ug/mL e) 400 ug/mL f) 1600 ug/mL g) 3200 ug/mL h) negative control
Figure 5.7 Prussian Blue Histochemical Stain to mark updating of iron nanoparticles, 20x magnification for varying nanoparticle concentrations: a) 1 ug/mL NP-Hep b) 10 ug/mL NP-Hep c) 100 ug/mL NP-Hep d) 1 ug/mL NP-NH$_2$ e) 10 ug/mL NP-NH$_2$ f) 100 ug/mL NP-NH$_2$ g) Control
Prussian Blue staining was used to determine the fate of nanoparticles after 2 hours. The Prussian Blue stain is a histochemical reaction that marks deposits of ferric iron in bright blue and is used to visualize cellular uptake of magnetite nanoparticles [32-34]. Seen in Figure 5.7, treatment with amine terminated nanoparticles produces a noticeable change in phenotype with the formation of many vesicles (indicated in red arrows). Close examination of cells treated with 1 and 10 µg/mL amine nanoparticle shows dark blue staining in the vesicles suggesting presence of nanoparticles. After only 2 hours of treatment, 100 µg/mL of amine particle proved toxic leading to fragmentation of cells, which resulted in an absence of cells in images. Heparin nanoparticles at any tested concentration do not appear to produce any phenotypic change observable with phase contrast microscopy.

Alpha smooth muscle actin is one of the markers commonly used to indicate a VSMC lineage towards either the synthetic or contractile phenotype [35-37]. VSMCs in the contractile phenotype have a spindle shape with contractile apparatuses aligned into fibers [38]. We observed the alpha smooth muscle actin content of VSMCs using immunofluorescence, Figure 5.8. It is immediately obvious that the control amine terminated particle disrupted normal cell behavior resulting in cell fragmentation. Interestingly, this process seems to remove even the nuclei (shown in blue). The synthetic phenotype can be observed in the Figure 5.8.a with a rhomboid shake and randomly oriented fibers. VSMCs subjected to heparin nanoparticles appear to have a more organized actin distribution and assume the spindle shape associated with the contractile, quiescent phenotype.
Figure 5.8 Immunofluorescent staining of alpha smooth muscle actin (green) and nuclei (blue) stained with DAPI, 20x magnification for varying nanoparticle concentrations: a) 0 ug/mL b) 1 ug/mL NP-Hep c) 10 ug/mL NP-Hep d) 1 ug/mL NP-NH₂ e) 10 ug/mL NP-NH₂
5.5 Discussion

Before heparin coated magnetic nanoparticles can be used in a clinical setting, there must be many tests to determine the optimal dosing concentrations and their effects. This study shows a first step towards this goal. As mentioned in the results, two different batches of nanoparticles were used in this study, and the loading of heparin per nanoparticle varied widely from batch (see Chapter Four for full details of heparin concentration determination). Heparin nanoparticles in the Live/Dead study were loaded with 2.8896 µg / µg of nanoparticle, while all other studies were loaded with 0.976 µg of heparin / µg of nanoparticle. Heparin has one of the highest charge density of any known biological molecule due to the multitude of sulfate and carboxylic acid groups [39]. This 3-fold difference has the potential to leave a huge impact on the results of the studies.

The first step to evaluation of the potential for use in the clinic is determination of anti-proliferative/cytotoxic effects. In this study, we used MTS and Live/Dead assays to measure these characteristics. As shown in Figure 5.1, the anti-proliferative effects of heparin nanoparticles appear to follow a dose dependent fashion, which is indicative of potential future use. Unfortunately, the Live/Dead results do not match up with the MTS results. As seen in Figure 5.2, the Live/Dead study shows a reversal of the trend shown from the MTS assay. The previously explained excess heparin loading could explain their toxicity. The plasma membrane of a cell is covered with proteins, which require a specific conformation to function. One possible explanation is bringing a loaded heparin nanoparticle (ie. a highly negatively charged particle) in contact with many surface
proteins at once results in disruption of many membrane proteins, which could trigger cell death.

Further evidence for this theory is supported by the fact that heparin alone did not shown any inhibition of proliferation at up to 3.2 mg/mL. Previous studies have shown significant changes in cell proliferation at concentrations as low as 25 µg/mL [31]. Although, it has been shown that the effects of heparin are dependent on the commercial brand of heparin [40]. Partial explanation for this variance comes from the fact that it has been shown that the anti-proliferative effects of heparin depend on very complex mechanisms involving the degree and location of sulfate groups [41].

Characterization of the fate of the nanoparticle is an important aspect of biocompatibility studies. Through Prussian Blue in Figure 5.7, we see support for the cellular uptake of amine particles. This appears to be consistent with other work that shows positively charged nanoparticles have greater cellular uptake than negatively charged particles [42]. Our results support a previous hypothesis that more positive nanoparticles have a greater affinity to the negatively charged cell bilayer membrane [28] causing to increased interactions and toxicity.

Based on the toxicity data (MTS), the optimal dosing appears to be at 10 µg/mL nanoparticle (1 µg/mL heparin) where we observed a 40% reduction in relative cell proliferation. A previous study has required 1600 µg/mL heparin to achieve this level of anti-proliferative effects [31]. It is plausible that proliferation inhibition occurs at this lower dosing than typical literature values due to the increased localized concentration from a single nanoparticle interacting with a cell. From Chapter Four, we know that 1
nanoparticle contains approximately 7,300 heparin molecules. Therefore, we may have achieved induction of a possible phenotypic shift observable by the distribution of alpha smooth muscle actin stained in Figure 5.8. While quantitative measurements were outside the scope of this study, there appears to be an increase in the number of spindle shaped cells with aligned actin fibers after treatment with 1 µg/mL and 10 µg/mL heparin nanoparticle. These features are indicative of the contractile phenotype and suggest that the use of heparin nanoparticles may be tool in the prevention of neointimal hyperplasia.

5.6 References


[38] D. M. Milewicz, C. S. Kwartler, C. L. Papke, E. S. Regalado, J. Cao, and A. J. Reid, "Genetic variants promoting smooth muscle cell proliferation can result in


CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Clinicians have been battling neointimal hyperplasia for decades. Significant gains have been made into understanding the underlying mechanism. There has been clinical progress with the acceptance of drug eluting-stents. However as it stands with current technology, stents have problems leaving the room open for innovative solutions. Nanoparticles have shown great promise in a variety of applications. In particular, magnetic nanoparticles have shown great promise for the biomedical field. This research describes the application of magnetic nanoparticles to inhibit neointimal hyperplasia.

In our first study, we demonstrated successful synthesis of a magnetic nanoparticle tailored to a specific range. The importance of nanoparticle size in biomedical applications cannot be underestimated, and we consider the nanoparticle size distribution between 20 and 30 nm to be a great success. Heparin was chosen as the drug of interest as it induces duel effects of anticoagulation and antiproliferation. We successfully attached heparin to a nanoparticle that we have previously shown to be biocompatible. Although, variability in the synthesis process led to differences in amount of heparin loaded on each batch of nanoparticles, the results successfully demonstrate the capacity to create a heparin coated magnetic nanoparticle.

Our second study describes the initial testing to determine VSMC proliferation and toxicity in response to the synthesized particles. An ideal outcome would be a
reduction in cell numbers without induced cell death. Rather, VSMCs would undergo a phenotypic shift from the synthetic to the contractile phenotype. Depending on the batch of nanoparticles used, we observed either inhibition of proliferation or cell death, which we attribute to the 25 fold difference in heparin loading between the batches. This study is promising in that the low concentration of heparin nanoparticles led to a reduction in cell number without any noticeable negative change in cell morphology. Rather, we observed a possible change in actin distribution, which would be indicative of the contractile phenotype.

6.2 Recommendations

Taking both studies together, these results demonstrate successful synthesis of heparin-coated nanoparticles and give indication for future investigation. Removal of heparin loading variability seen in our studies should be the first step for future work. Our results support that the effects of heparin nanoparticles will be concentration dependent; therefore, it will be critical to determine the optimal dosing range to inhibit proliferation. It is likely that this range will depend on the concentration of both bound heparin and nanoparticles in solution. Furthermore, the phenotype of VSMCs after treatment will need to be assessed to determine if this treatment is likely to produce long-term effects by induction of the contractile phenotype. The phenotype change should be characterized in terms of gene/protein expression and mechanical properties in terms of Young’s Modulus.
To fully evaluate the effects of these nanoparticles, it will be important to add studies characterizing the effects of alcohol terminated particles following the ligand exchange in Chapter Four. Furthermore, it should be noted that all particles should be run through the water column immediately prior to cellular testing. In addition, the effects of these particles will need to be evaluated on other cell lines, particularly endothelial cells due to their proximity to VSMCs and cells that can be found throughout the body (ie. fibroblast) to evaluate potential systemic toxicity.

The whole premise of this treatment relies on the ability to keep these magnetic nanoparticles concentrated while under pulsatile blood flow. This capability will depend largely on the synthesis of the nanoparticles; therefore, it may be necessary to adjust the synthesis method to attain the optimal particle size. With the optimal particle, the efficiency will have to be evaluated in animal studies. There remains much to be understood before this treatment can be used in a clinical setting; however, the results of this study suggest that it may one day see clinical application.
APPENDICES
Appendix A: Fe Determination Protocol – Up to 400 µg Fe

(Revised 2/5/14)
6th Revision

Iron Solution Preparation:
1. Obtain desired iron sample for test.
2. Transfer 5-8 µL for concentrated samples or 100 µL for dilute samples to a 10 mL volumetric flask with a calibrated 10 µL pipette. Note: Use one of the short-necked flasks to allow the pipette to reach the bottom of the flask.
3. Dissolve the iron in the flask with approximately 0.2mL of concentrated HCl.
4. Once all the iron has dissolved (about 15 sec), fill the volumetric flask with deionized water to the 10 mL mark. This is the iron stock solution. Using the ground glass stopper, mix by inverting the 10 mL volumetric flask 3 times.
5. Transfer the solution from the volumetric flask to a disposable 15 mL centrifuge tube. Make sure the solution is well-mixed.

Sample Preparation:
*Note: Glassware is stored in the drying oven and should be rinsed at least 2X with deionized water following use.
1. Using the calibrated 1 mL pipette, transfer 0 (blank), 0.5, 1, 1.5, 2, and 2.5 mL of iron stock solution to several 15 mL centrifuge tubes. Label each centrifuge tube with these amounts. These may vary. Blank will include the following solution with the exception of iron.
2. Dilute the iron solution in each centrifuge tube to approximately 6 mL with deionized water.
3. To develop the color:
   a. Add 0.2 mL of hydroxylamine hydrochloride solution\(^1\) to each centrifuge tube and swirl to mix.
   b. Add 0.5 mL of 1,10-phenanthroline solution\(^2\) to each centrifuge tube. Mix.
   c. Add 0.75 mL of the ammonium acetate-acetic acid solution to each centrifuge tube (including the blank).
   d. Bring the volume of each centrifuge tube to 10 mL with nanopure (DI) water using a pipette. Mix well by inverting the centrifuge tubes.
4. Transfer about 3 mL from each flask to disposable PMMA cuvettes. Transfer the blank to two cuvettes to create a blank for UV/Vis measurements.
Measure Absorbance:
1. Measure all samples using the UV-Vis spectrophotometer
2. For each sample record the absorbance at 511nm (the Fe peak) and at 690nm (the baseline)
3. Once recorded, subtract the 690 nm absorbance from the 511 absorbance. This will be the value you insert for your absorbance into the iron determination spreadsheet.

Cleaning the Volumetric Flask:
1. After stock solution is made and transferred to a 15 ml centrifuge tube, add about 3 ml of conc. HCl to the volumetric flask.
2. Swirl it around for about 30 seconds and dispose of the acid.
3. Rinse 2x with the water in the fume hood.
4. Bring out of the fume hood and rinse 2x with DI water.
5. Place back in drying oven for the next person to use.

*Note: If a number does not pass the Grubb’s test in Excel Iron Determination:
   a. Re-enter the number in the column for the Grubb’s test in order to eliminate original equation.
   b. Delete corresponding number in the µg/100mL column.
   c. Highlight the row in red.

*Note:
1. Hydroxylamine hydrochloride solutions (100 g/L)—dissolve 1 g of hydroxylamine hydrochloride in 10 mL nanopure water.
2. 1,10-phenanthroline solution (3 g/L)—dissolve 0.3 g of 1,10-phenanthroline monohydrate in 10 ml of methanol and dilute to 100 ml with nanopure water.
3. Ammonium acetate-acetic acid solution—dissolve 10 g of ammonium acetate in about 60 mL of water, add 20 mL of glacial acetic acid, and diluted to 100 mL with nanopure water.

^a Method Title “Fe Determination” subject to change. Reference: ASTM, E 394-0
Appendix B: DMMB Assay

Written by: Alex Lindburg-MBEM Lab
Updated: Elliott Mappus 3/4/15

Materials
1. Chondroitin sulfate (Sigma C4384)
2. 1,9-dimethylmethylene blue reagent (Sigma 341088) [See Notes 1 & 2]
3. Glycine
4. NaCl
5. HCl
6. 96-well plates [with U-shaped bottoms]

Directions
1. 20 µL aliquots of media in triplicate should be used for analysis.
2. The sample with the GAG highest concentration should be tested so an appropriate dilution can be made [See Note 3].
3. A standard curve should be made for comparison of media to solutions of known chondroitin sulfate concentrations. Make a working solution of 100 µg/mL by adding 2.0 mg of CS powder to 20 mL of PBS.

<table>
<thead>
<tr>
<th>Working Solution (µL)</th>
<th>PBS (µL)</th>
<th>Final Concentration (µg/µL)</th>
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<tr>
<td>0</td>
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<td>0</td>
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<tr>
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<td>200</td>
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4. 180 µL of DMMB is added to each well using a multi-channel pipet
5. Shake samples for 5 seconds
6. The absorbance at 530 nm is measured immediately with a microtiter plate reader.

Notes
1. The working solution is made by dissolving 16 mg of DMMB dye in 1 L of water containing 3.04 g glycine, 2.37 g NaCl, and 95 mL of 0.1 M HCl. Filter the solution through Watman paper. Do not use if there is precipitate in it.
2. The solution should be stored at room temperature in a brown bottle. The solution is stable for up to 2 months.
3. The linear concentration of chondroitin sulfate in DMMB is limited to between 10 and 50 µg/mL (0.2 and 1.0 µg in 20 µL of solution).

Reference
Appendix C: DMMB Standard Curves

Comparison of heparin and chondroitin sulfate standard curves. The difference in slopes was found to be statistically insignificant ($P > 0.4$).
Appendix D: Isolating Vascular Smooth Muscle Cells

1. Sacrifice rat using CO\textsubscript{2} asphyxiation and cervical dislocation

2. Remove aorta from subclavian origin to the diaphragm bifurcation and place in DMEM with pen/strep

3. Use tissue culture hood to remove all adventitia and connective tissue. All adventitia must be removed to ensure you only attain VSMCs

4. Cut vessel longitudinally so it lays flat and scrape off endothelium gently with scalpel blade and rinse thoroughly in DMEM with pen/strep

5. Cut the vessel into ~5 mm squares and place them in digestion solution that includes 5 ml DMEM, pen/strep (1%), Collagenase type II (final concentration 175U/ml), and 10% FBS for 20 minutes

6. Centrifuge at 500 rpm for 1 minute and remove the supernatant and wash the pellet with DMEM containing pen/strep

7. Resuspend the pieces in another digestion solution containing DMEM, pen/strep (1%) Collagenase type II (final concentration 175U/ml), Elastase type III (final concentration 0.25 mg/ml) and 10% FBS for 1 hour. Be sure to shake the tube gently every 10 minutes until the vessel is gossamer thin. When the vessels are dissolved stop the digestion by diluting with equal volume of DMEM with pen/strep and 20% FBS

8. Centrifuge 1000 rpm for 5 minutes and remove the supernatant

9. Suspend cells in DMEM with pen/strep and 20% FBS and place in T-25 flask and put in incubator (37\textdegree C, 5% CO\textsubscript{2}). Don’t change media for 72 hours as cells need
sufficient time to adhere to flask.

10. Maintain the cells using DMEM with 20% FBS and pen/strep until cells reach P4 then use 10% FBS