Fabrication and Manipulation of Micro- and Nano-Scale Magnetic Particles: Application to Magnetofection, Nanopositioning, and Drug Delivery

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ABSTRACT

(Under the Direction of Richard Superfine.)

Magnetic particles offer scientists a unique opportunity – the ability to apply forces to these particles from a distance by means of a magnetic field gradient. This property becomes increasingly useful for probing and prodding physical processes in the biological world. Because a large fraction of biological materials are non-magnetic we are able apply forces to the particles which are not felt by the biological samples, unless of course the biomaterials are in some way mechanically coupled to the magnetic particles. This dissertation describes two techniques for creating shape-defined and compositionally flexible magnetic micro/nanoparticles. Both techniques fall into the category of top-down synthesis, making use of templates to pattern and grow particles. The first technique relies on thermal evaporation of metals onto pre-patterned wafers, the resulting metal deposition creating clusters of material atop lithographically defined posts. The technique produces shape-defined particles and allows for user-defined particle composition (resulting materials named Post-Particles). The second technique relies on electrodeposition into the pores of anodized aluminum oxide templates and results in aspect ratio-adjustable magnetic rods with a wide range of diameters. Here I use the technique to create novel nickel-gold Janus nanorods. In this dissertation I apply particles created using these
techniques to three different biological scenarios. First, I demonstrate the process of enhanced oligonucleotide delivery to cells in vitro using magnetic Post-Particles delivered to cells in an applied magnetic field gradient. Next, high aspect ratio Janus rods are implemented as rotational swimmers capable of cargo and single cell manipulation in microfluidic settings. Finally, magnetic nanorods are applied to the process of nanoparticle transport through protein-rich gels and their motion is quantified on a single-particle basis. This final experiment is designed to inform the community of researchers interested in magnetic drug targeting as to the forces experienced by and types of motion demonstrated by rod-shaped nanoparticles moving through extracellular matrix, the primary barrier to long-range nanoparticle distribution in localized cancer tumors. This chapter presents the first data on non-continuous motion (moving in fits-and-starts) of small particles undergoing magnetophoresis through the extracellular matrix, and offers this data in direct contrast with continuous motion of large particles through the same material.
Acknowledgements

I have enjoyed this opportunity to do science with nanoparticles and magnets, and at times it has succeeded in blurring the lines between work and play. Indeed, I have garnered great pleasure from watching particles which started out unformed macroscopic metal chunks (thermal evaporation seed material) or ions in solution (electrolytes used in template-guided electrodeposition) become active agents with a bit of electricity and a magnetic field. Watching these creations as they deliver small molecules to cells, swim towards and capture beads, or dance their way through a complex protein meshwork has been truly fulfilling. Through this research I have discovered that, as an experimentalist, there are no greater tools than one’s imagination, access to the work done by prior scientists, and a friendly set of knowledgeable collaborators.

I have had the perhaps unusual experience of enjoying graduate school a great deal. Naturally, there were very trying times, and rather easy times. Throughout the spectrum of graduate school experiences there have been several people without whom I absolutely never would have continued the whole endeavor.

First I would like to thank my committee. Rich, your ability to direct a research group with such energy and devotion is impressive, to say the least. Your knack for asking the questions that immediately get to the heart of interesting science is enviable. I sincerely thank you for your time, your financial support, and your
patience. To Sean I owe a great debt for initially giving me a chance at graduate school and for always providing honest, uncensored insight into the mysterious world of professional science. Your insistence on merciless candidness in matters scientific and personal have meant more than you may imagine. Thanks for all this, as well as the occasional free burrito. To Otto, Joe, and Lu-Chang: Thank you for taking the time to advise, despite your very busy schedules. I am indebted to Amy Oldenburg for sitting in on my defense in a pinch – I am very grateful!

Naturally, I must also thank the funding agencies which made my time in graduate school (financially) simple. I would like to thank the UNC Alliance for Graduate Education and the Professoriate for funding my first year of graduate school, and the The Carolina Center of Cancer Nanotechnology Excellence for funding some of the work in between my first year and my sixth year. I would especially like to thank Ross and Charlotte Johnson for their generous support to the UNC Royster Society of Fellows Program. A dissertation completion fellowship through this program funded my last year of graduate school, and for this I am truly grateful.

As for my family, my parents have been ever-supporting, ever-confident, and ever-loving. That second attribute, the persistent confidence in me, often substituted for the utter lack of confidence I had in myself. I will always be grateful for this. Thanks Mom and Dad. My brothers too have contributed to my stability more than perhaps they would have imagined. Dewey: your stubborn and obnoxious challenges, both scientific and personal, have always given me pause, and have been with me more than you may imagine during my time in the lab. Cavan: your relaxed
attitude is infectious and there have been several times when thinking back to this has provided a much-needed calming.

For whatever reason, I never expected to love work when I grew up. Perhaps I will never love work as much as I have loved my time in NSRG. I hope not, but it wouldn’t surprise me if this ended up being the case. The people of NSRG truly made doing graduate work in our lab memorable, enjoyable, and profitable, on both personal and scientific levels. Many of you became friends above colleagues. To Adam Hall, for taking me under his wing during my first few timid days in our lab, and for turning that mentorship into a wonderful friendship. To Adam Shields, for always asking the difficult question. To Jerome Carpenter, ever-present bearer of a smile and a healthy dose of perspective. To Kwan Skinner, thanks for the many lessons in electrodeposition, perseverance, and the value of randomly musing about potential experiments; I always enjoyed our conversations, whether practical or fantastical. To Benjamin Evans, modeler of magnetic things and constant bearer of back-of-the-envelope calculations and the correct equation for a given situation, thanks for your work in modeling the swimming behavior of rotating rods. To Jeremy Cribb, Xiomara Calderón-Colón, Brian Eastwood, Briana Fiser, Kris Ford, Nathan Hudson, Ricky Spero, Vinay Swaminathan, Nadira Williams: thanks for making graduate school, and time in NSRG, as wonderful as it was.

Also, Mike Falvo deserves a special thanks for his insightful questions about making things at the micro- and nanoscale and general advice on doing science. As does Tim O’Brien for his oversight of microscopes and lab biology. Kevin Herlihy and
Janine Nunes deserve a special thanks for including me in a project involving their magneto-polymer composite particles. The amazing team which operates the Chapel Hill Analytical and Nanofabrication Laboratory (CHANL) is a crucial component to all micro- and nanoscale research (and materials science research in general) going on at UNC. Many thanks to Carrie Donley, Amar Kumbhar, Wallace Ambrose, and Bob Geil for their assistance in microscopy, sample preparation, thin film deposition, and many other experimental procedures which have gone into this thesis. Also, on the microscopy front I owe a great debt to Michael Chua and Neal Kramarcy of the Michael Hooker Microscopy Facility (MHMF), as they kindly took me under their wing and showed me the intricacies of performing fluorescence recovery after photobleaching experiments (Chapter 5).

Finally, there is my Only – that one person whom, after meeting, all of life changes. Christy, going to graduate school is, I imagine, generally a good decision. Going to graduate school with you has been nothing short of magical. It’s funny to realize we have spent the entirety of our relationship with both of us in school. I can think of no better way to set sail on the high seas of life. Thank you so much for sharing every graduate school success, and more importantly, for softening every demoralizing graduate school bungle. I am ever-proud of you, ever-impressed by you.
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<td></td>
</tr>
<tr>
<td>AAO</td>
<td>anodized aluminum oxide</td>
<td></td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscope</td>
<td></td>
</tr>
<tr>
<td>DI</td>
<td>deionized</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
<td></td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
<td></td>
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<tr>
<td>EDS</td>
<td>energy dispersive x-ray spectroscopy</td>
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<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
<td></td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
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<tr>
<td>HBE</td>
<td>human bronchial epithelial</td>
<td></td>
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<tr>
<td>IM</td>
<td>interstitial matrix</td>
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</tr>
<tr>
<td>IPA</td>
<td>isopropanol</td>
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<tr>
<td>IPN</td>
<td>interpenetrating network</td>
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<tr>
<td>MDT</td>
<td>magnetic drug targeting</td>
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<tr>
<td>MNP</td>
<td>magnetic nanoparticle</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>NP</td>
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<tr>
<td>NR</td>
<td>nanorod</td>
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<tr>
<td>NW</td>
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<tr>
<td>ODN</td>
<td>anti-sense oligonucleotide</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
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</tr>
<tr>
<td>PCTE</td>
<td>polycarbonate track etched</td>
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<tr>
<td>PDMS</td>
<td>poly-dimethylsiloxane</td>
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<td>PEG</td>
<td>poly-ethylene glycol</td>
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<td>PPO</td>
<td>poly-propylene oxide</td>
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<tr>
<td>PS</td>
<td>polystyrene</td>
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</tr>
<tr>
<td>Re</td>
<td>Reynolds number</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscope / microscopy</td>
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<td>TEM</td>
<td>transmission electron microscopy / microscopy</td>
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## List of Symbols

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<td>$\mu_0$</td>
<td>relative permeability of free space</td>
</tr>
<tr>
<td>$R_H$</td>
<td>radius of hydration</td>
</tr>
<tr>
<td>$r_p$</td>
<td>pore radius</td>
</tr>
<tr>
<td>$r_s$</td>
<td>solute radius</td>
</tr>
<tr>
<td>$\phi$</td>
<td>volume fraction</td>
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<tr>
<td>$\eta$</td>
<td>viscosity</td>
</tr>
<tr>
<td>$d_f$</td>
<td>fiber diameter</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>the ratio $R_H/r_p$</td>
</tr>
<tr>
<td>$\omega$</td>
<td>angular frequency of a rotating magnet</td>
</tr>
<tr>
<td>$\omega_{\text{bleach}}$</td>
<td>radius of bleach spot</td>
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A note regarding embedded videos in this thesis

This thesis contains videos. Specifically, Chapter 5 and Chapter 6 contain videos demonstrating various types of magnetically induced particle motion. Unfortunately, these videos will generally only be viewable using Adobe Acrobat Reader (Version 9+ should be sufficient), on a computer which has Quicktime installed (both of these are freely available). Video locations will first appear as large blank swaths of space on a given page, with the accompanying text “Video: Description of video contents.” Clicking in this area should initiate video playback. Your reader may ask you if you trust the document first. Also, alongside the video I have included a still image representing what the video generally attempts to demonstrate. If the version you have acquired does not seem to contain the embedded videos and you would like a version which does, feel free to send a request to lamar.mair@gmail.com.
Chapter 1

Introduction

Magnetism has fascinated and enabled us for millennia, certainly long before we had any understanding of its mechanism. Interestingly, the medical implications of magnetism are some the oldest, dating back to a Sanscrit medical text written circa 800 BCE, the Sushruta Samhita, which reported the surgical procedure of removing metallic splinters via the application of a magnetic force (Sarton, 1927; Vowles, 1932).

1.1 Thesis statement and contributions

This thesis argues the usefulness of inorganic, ferromagnetic micro- and nanoscale particles for experiments involving cells and the material surrounding cells – the extracellular matrix. The two principal efforts produced in this research are (1) the fabrication of inorganic, ferromagnetic micro / nanoscale particles, and (2) applying magnetic fields for manipulating these particles in various biological settings in vitro. Two different methods are used for making magnetic particles (discussed in Chapters 3 and 4), both relying on types of templates for defining particle size and
shape. It should be mentioned that, while this thesis focuses on inorganic particles and elaborates on some of the advantages of these particles, I am fully aware of their (often severe) limitations when compared to polymer-based particles. That said, two of the experiments presented in this thesis rely on the advantages of these inorganic particles, primarily the high magnetization exhibited by these particles. As will be discussed later, both experiments on single cell rotational manipulation and magnetically induced particle transport through extracellular matrix depend on particles having high magnetization. Additionally, this thesis contributes to an overall understanding of the commercial extracellular matrix Matrigel. In Chapter 5 I show that macromolecular diffusion in Matrigel (for particles with radii on the order of 2–25 nm) is very similar to literature reports for the ECM in vivo, as well as other reports on other matrices in vitro. Chapter 5 represents the first time in which a single Matrigel diffusion data set has been analyzed using the Renkin, Ogston, and Phillips models concurrently.

1.2 Outline

This work is outlined as follows:

Chapter 2 introduces magnetophoresis, explains the basis for its implementation, and compares magnetophoresis with several competing techniques. A few niche magnetophoresis applications are discussed, specifically within the realm of biomedical nanotechnology. Previous work in the fields of magnetofection, magnetic
cell manipulation, and particle transport through polymers is briefly surveyed.

Chapter 3 demonstrates a template-based technique for making size- and shape-engineered, multi-metallic particles. Particle characterization is performed with respect to particle composition, and the magnetic properties of Fe particles grown using this technique are determined via magnetophoretic transport through a liquid in a known magnetic field and field gradient. This chapter ends with a demonstration of successful magnetofection using these particles.

In Chapter 4 the technique of templated electrodeposition is used to create multisegmented magnetic rods. Additionally, this chapter presents a fabrication scheme for creating the first magnetic Janus nanorods, and utilizes these rods in rotational near-surface swimming. First demonstrations of payload capture using a swimming Janus rod is demonstrated, as is a first demonstration of directed single cell manipulation via rotation near a surface.

Chapter 5 introduces the extracellular matrix (ECM) as a material, discusses its structure, and relates its importance in tumor biology. Specifically, the process of macromolecule (diffusive) transport through a cell-culture-derived ECM (BD Bioscience’s Matrigel) is discussed. Making use of the fluorescence recovery after photobleaching technique, the size-specific diffusion suppression for a range of dextrans with varying molecular weights is shown and compared with other studies of extracellular matrix materials. Basic models of the matrix are discussed and the experimentally determined values for diffusion coefficients are compared with theoretical values obtained via these basic models. New insight into how the models
predict diffusion indicates that the Phillips model overpredicts diffusion suppression in Matrigel, information which has not previously been established for Matrigel.

In Chapter 6 nanorods are magnetically driven through Matrigel. The work detailed in this chapter represents the first single particle tracking experiments of rod-shaped particles moving through ECM. Rod velocities are quantified, and the ratio of velocity/applied force is compared for a set of particles with known dimensions. Single particle tracking observations of two distinct modes of transport for small versus large particles demonstrates a balance between force and size and shows that smaller forces on some particles (smaller particles) may result in significantly larger average transport velocities. Also, the single particle tracking of small particles demonstrates the significance of diffusion in the magnetophoretic process: small particles which experience small forces were able to move through matrix pores (more effectively than larger particles experiencing larger forces) because diffusive forces acted on them in an appreciable way, allowing them to diffuse out of the path of the matrix material preventing them from making forward progress.

Finally, Chapter 7 offers a brief conclusion of the presented work, as well as future directions.
Figure 1.1: General theme of this dissertation.
Chapter 2

Overview of magnetophoresis

Magnetophoresis is the motion of magnetic particles relative to a fluid, as induced by an applied magnetic field. Broadly, the motion can be used to separate magnetic particles from non-magnetic particles (Watarai et al., 2004). This application of magnetic force has wide-ranging applications and, historically, has been used for mineral separation (Oberteuffer, 1973), water treatment/remediation (Kakihara et al., 2004), synthetic molecule separation (Moeser et al., 2002), protein separation (Bucak et al., 2003), continuous DNA separation (Karle et al., 2010), and cell sorting (Miltenyi et al., 1990; Pamme and Wilhelm, 2006; Pamme, 2006). The concept is equally applicable to the manipulation of large groups of particles and single particles: Anker et al. succeeded in manipulating, tracking, and characterizing swarms of ferromagnetic particles (Anker et al., 2007), while Fan et al. demonstrated single nanorod magnetophoresis to deliver cytokines to individual cells with sub-cellular resolution (Fan et al., 2010).

The definition of magnetophoresis is not strict and the emphasis is on the fact that particles move relative to a surrounding fluid — that is, they are translated some
distance. Typically, magnetophoretic motion is induced by a magnetic field gradient, and quantitative translation using this technique is demonstrated in Chapter 3. Additionally, in Chapter 4 I include experimental work on hydrodynamic swimming induced by magnetic rotation as a form of magnetophoresis, as the result is translation through a solution. This translation method is possible in the absence of a field gradient.

An added capability of magnetofection is that of magnetic particle property characterization. For systems in which (1) particles are dispersed in a Newtonian solution of a known viscosity, (2) the particle shape and volume are well known, and (3) the applied field and field gradient are known to a high degree, the resulting magnetofection can be useful in characterizing the magnetic properties of the particles involved (Zhang et al., 2005; Mair et al., 2009). The ability to be highly quantitative with respect to the relevant forces during magnetofection experiments is an underlying theme of this work in general and is discussed in Chapters 3, 4, and 6.

2.1 Magnetophoresis and competing techniques

There are myriad competing techniques for manipulating micro- and nanoscale objects in solution. The principal methods used for performing manipulations in a fluidic environment utilize optical (Ashkin et al., 1987; Grier, 2003), electrical (Becker et al., 1995; Pethig, 1996; Pethig and Markx, 1997; Huang et al., 2001), acoustical (Groschl, 1998; Neild et al., 2006; Neild et al., 2007), and magnetic (Yellen et al.,
fields and field gradients for applying forces.

Optical techniques for manipulation (commonly referred to as optical tweezers or optical traps) were first implemented by Ashkin in the mid-1980’s (Ashkin et al., 1986). Ashkin observed that the force impinging on a particle in the path of an optical beam could be enough to offset the gravitational force on the particle. Skeletally, a tightly focused laser beam contains a strong electric field at the beam waist, the narrowest point of the beam. The field gradient is highest near the center of the beam, and dielectric particles are attracted to the high intensity region, with the gradient providing the driving force. One significant advantage of optical tweezers is that, once implemented, they are capable of object handling in three dimensions. While optical tweezer setups are capable of manipulating objects ranging in size from one nanometer to hundreds of micrometers, the range of forces capable of being applied remains relatively small – on the order of hundreds of piconewtons (Simmons et al., 1996; Dao et al., 2003; Li et al., 2009). An additional drawback is that many samples, when trapped with a laser for long periods of time, begin to accumulate excessive thermal energy. In biological settings this can negatively impact the trapped particle, the surrounding environment, or both (Peterman et al., 2003). A final drawback of optical traps is that they typically require entire table-sized optical setups which are expensive, space-consuming, and difficult to implement in point-of-care applications. Magnetophoresis setups only require a microscope and magnets. Permanent magnets are ubiquitous, inexpensive, and easy to carry.
Electrical manipulation techniques, such as electrophoresis and dielectrophoresis, involve the translation of dispersed particles in a solution due to the application of a spatially uniform electric field. The electrophoretic effect was first observed by Reuss in 1809 in an experiment during which clay particles suspended in water displayed clear migration behavior upon applying a constant electric field (Reuss, 1809). Electrical manipulation relies on the existence of an electric surface charge, the applied electric field acting on this surface charge via an electrostatic Coulomb force. Double layer theory requires that surface charges in fluids are screened by a double layer of ions, this double layer having the same magnitude and opposite sign as the particle surface. Therefore, the force applied by the field is, technically, applied to the diffuse double layer surrounding the particle, and the force is passed along to the particle. Generally speaking, electrophoresis is capable of manipulating objects from one nanometer up to hundreds of micrometers. Relative to optical techniques, electrophoretic methods are generally capable of applying larger maximum forces, up to hundreds of piconewtons or even tens of nanonewtons, in some cases. Additionally, electrophoretic force application can typically be accomplished using a localized potential generated by electrodes in the near vicinity of the sample. Also, while optical manipulation typically requires dielectric particles, electrical methods of particle manipulation can be applied to a wide range of particle-solvent mixtures, including conductive particles in dielectric solvents as well as dielectric particles in conductive solvents. However, electrical manipulation may also result in heating and / or charging of the particle, which can have significant and detrimental effects
on the particles, cells, or organisms being manipulated, and may also energetically impact the physical relationship between the manipulated object and its surroundings (Grahl and Märkl, 1996).

Figure 2.1: Crossbar arrays of individually addressable microelectrodes provide an efficient, simple method of manipulation (Lee et al., 2007).

One of the more recent developments in particle manipulation lies in the area of acoustics. Acoustical manipulation relies on generating standing waves in a sample material containing particles. The standing wave creates a landscape of regions with varying acoustical pressures. The particle response to the applied standing wave is to move to areas of minimal acoustic pressure via the so-called primary acoustic radiation force. The two most common geometries for acoustical manipulation are the plane standing wave, which generates equidistant parallel planes and corresponding acoustical pressure minima, and the cylindrical wave geometry, which generates pressure concentric rings of low pressure (Groschl, 1998; Gherardini et al., 2005). Recently, Petersson and Laurell have demonstrated a variety of separations in microfluidic chips for on-chip separation of lipid particles from cells (Petersson et al.,...
2005; Laurell et al., 2007). The acoustical manipulation method relies on differences in the mechanical properties (density and stiffness) of the particles relative to the surrounding fluid environment. While acoustical manipulation has the benefit of not requiring localized probes to apply pushing or pulling force to the object of interest, it suffers from a relatively low maximum force and a broad range of potential particle locations. Additionally, pressure nodes tend to be broad and, due to low force application, manipulation of very small particles is sometimes impossible due to the comparatively large Brownian motion effects these small particles experience.

Figure 2.2: Red blood cells separated using acoustical manipulation (Gherardini et al., 2005).
Magnetic manipulation techniques offer a method for controlling essentially any object larger than 1 nm. Additionally, magnetic manipulation techniques are capable of applying high force, as the force applied is a function of the volume of magnetic material involved in the system. Magnetic control techniques typically do not involve charging or heating of the sample (of course, ignoring the field of hyperthermia in which heating is desirable). Moderate magnetic fields have the added benefit of being biologically and, in most cases, chemically invisible (Adair, 1991). And while magnetic manipulation can frequently make use of easy-to-use and readily available permanent magnets, the major drawback is that magnetic material must be present in a sample for manipulation to be successful.

2.2 Biomedical applications of magnetophoresis

This section introduces the subset of magnetofection applications which pertain to the material covered in subsequent sections of the document.

2.2.1 Application 1: Magnetofection

Gene therapy in vitro is, typically, a diffusion limited process: relevant therapeutics are delivered, often attached to liposomes, and diffusion is allowed to occur until the cells have received a sufficient amount of the therapeutic to understand and quantify its effectiveness. In the most simple application, diffusion is the only force acting on the vectors. This is particularly true for therapeutic vectors at the nanoscale,
which is commonly the case for simple virus- and liposome-based vectors. While agitation (mixing) may increase the frequency of cell-vector interactions, there is no guarantee that cells have enough time to effectively bind and endocytose the vectors in a system under constant agitation. Consequently, other more targeted physical methods have been developed for accelerating therapeutic vector concentration in the vicinity of cells. Bunnell et al. used centrifugal force for high-efficiency retrovirus mediated gene delivery to peripheral blood lymphocytes (Bunnell et al., 1995), invoking centrifugal force to increase gene delivery. Later, Luo and Saltzman used gravitational forces to allow settling and, consequently, accumulation of vector-DNA and vector-DNA-nanoparticle complexes at cell surfaces (Luo and Saltzman, 2000a).

Likewise, in vivo gene therapy relies on either diffusion or blood flow to carry the therapeutic throughout the system. These in vivo forces make utilizing and understanding externally applied forces all the more powerful, as their in vitro success paves the way for in vivo experimentation.

Magnetofection is the process of attaching a relevant biochemical payload to magnetic particles and delivering these loaded nanoparticles to cells directly by the application of a magnetic field. For the process to be successful the particles must (1) bind the relevant molecule / DNA / therapeutic, (2) land on or near the cell surface, (3) be endocytosed by the cell, and (4) allow for removal of the biochemical payload and delivery of this payload to the cell (indeed, it is possible that step (3), endocytosis by the cell, is not strictly required, as it is possible for the cell to remove the relevant payload from the particle without complete encapsulation of the particle). This
process was first demonstrated by Scherer et al. in 2002 (Scherer et al., 2002). In a sense, magnetofection is a biological application of magnetic nanoparticles which operates, at its smallest dimension, at sub-cellular length scales. That is, the process of endocytosis and molecule delivery to the cell all occurs at the cell membrane and inside the cell volume. While it is true that the active process of magnetofection (transport of the particles from solution to the cell surface) may operate over distances of hundreds or thousands of micrometers, at the smallest scale the result occurs inside the cell (typically in the nucleus). The effects of magnetofection are intrinsically played out at the subcellular level. The next section, single cell manipulation, moves up in length scale: magnetic cell manipulation deals with force application at the scale of whole cells (that is, in the regime of tens of micrometers).

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<th>Survey of DNA transfection methods adapted from (Luo and Saltzman, 2000b)</th>
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2.2.2 Application 2: Magnetic cell manipulation

The cell is a complex and elaborate micro-machine capable of performing an amazing range of functions and exhibiting an astonishing range of chemical and
mechanical properties. As such, an entire field of engineering has sprung up, the end goal being the directed manipulation of individual cells in solution. Straying from exclusively magnetic manipulation, the field boasts a variety of single cell manipulation techniques, including optical tweezers, mechanical tweezers (Hagiwara et al., 2010), dielectric force manipulation (Lee et al., 2004a; Xia et al., 2006; Lee et al., 2007), and internalized magnetic particle manipulation (Pamme and Wilhelm, 2006; Pamme, 2007; Rodríguez-Villarreal et al., 2011). Hagiwara’s mechanical tweezer setup is shown in the lower set of images in Figure 2.4, and shows a single cell being manipulated laterally, as well as rotationally. The upper set of images in Figure 2.4 shows magnetic trapping of cells attached to Ni nanowires (Tanase et al., 2005). Westervelt’s research group has produced several reports on trapping magnetic materials using lithographically fabricated, individually addressable, perpendicularly
arranged crossbar arrays of gold wires. Supplying current through the wires creates complex and user-defined microelectromagnetic matrix of magnetic fields. Using these fields the researchers were able to translate and trap cells bound to magnetic particles (Lee et al., 2004a; Lee et al., 2004b). Several reports have also applied magnetic fields to magnetic particle bound cells in microfluidic channel flows, showing that particles loaded with magnetic materials can be quickly and efficiently separated and, furthermore, that particle uptake can be quantified based on each individual cell’s response to the applied magnetic field (Pamme, 2006; Pamme and Wilhelm, 2006; Xia et al., 2006; Lee et al., 2007; Rodríguez-Villarreal et al., 2011). Additionally, Sakar et al. infused polymer microtransporters with magnetic nanoparticles (Figure 2.5) in order to create magnetically actuable cell-vehicles on the scale of hundreds of micrometers (Sakar et al., 2010).

2.2.3 Application 3: Magnetic drug targeting: Nanoparticle transport through polymers / biopolymers

In vivo, cells exist in a complex protein network, the extracellular matrix. While delivering relevant molecular therapeutics to cells via magnetofection lies on a smaller length scale (micrometers) than manipulating individual cells using magnetic fields (tens of micrometers), the native environment of cells offers an even larger-scale scenario (hundreds of micrometers all the way up to centimeters) in which magnetic particles are useful. Of course this isn’t to say that the ECM is not
Figure 2.4: Two methods demonstrating the manipulation of individual cells. The upper two images show arrays of lithographically defined magnetic micropads which were used to order cells bound with magnetic nanowires. The field gradient concentration at the tips of the micropods attracts the highly magnetic Ni nanowires, pulling the cells into the ordered array shown (Tanase et al., 2005). The lower four images show the highly controlled manipulation of an individual cell using a magnetically driven tweezer-like microtool (Hagiwara et al., 2010).
Figure 2.5: These three images show the highly controlled acquisition of an individual dead cell using a magnetic microscale actuator (Sakar et al., 2010).

an interesting material at the nanoscale – on the contrary, it is an extremely rich and diverse protein composite structure, and the details of the ECM structure are elabo-
rated upon in Chapter 5. However, the field of magnetically targeted tumor delivery seeks to operate on the length scales of the matrix. Because magnetic fields are essentially biologically invisible and can be applied from outside of the body, there is enormous potential for using magnetic fields to guide and concentrate drug-loaded magnetic particles at a specific location in the body (Lubbe et al., 1999; Alexiou et al., 2000; Lubbe et al., 2001). The primary benefits of this technology lie in being able to dramatically increase the local dose of drug at the site of a tumor, while simultaneously decreasing the systemic dose of the drug. A cartoon of the general process is shown in Figure 2.6, demonstrating delivery of drug-loaded particles to a supplying tumor artery and the application of a magnetic field gradient (pole shoe in Figure 2.6). Specifically, in tumors the extracellular matrix acts as a primary inhibitor of small molecule and nanoparticle transport. Research into quantifying the transport of various magnetic nanoparticles in this material seeks to elucidate the transport properties of groups of particles in an applied field. Recently, Ruenraroengsak et al. assembled a review titled “Nanosystem drug targeting: Facing up to complex realities,” in which the authors note several of the challenges posed to successful nanoparticle-based drug targeting. Within this review the authors state, in a section titled “Unmet needs and challenges:”

“A better understanding of the movement of nanoparticles in the complex environments of tissues, tumours, blood and lymph is essential for prediction of their behaviour. The influence of particle size, shape and flexibility is key (Geng et al., 2007). Shape matters. If flow matters then asymmetric flow is clearly different
from the flow of spherical particles, hence there is a need for better comprehension in this area.”

– (Ruenraroengsak et al., 2010)

Clearly there is a need for understanding how magnetophoretically transported particles travel within biological polymer materials. The relevant literature on magnetically induced transport of nanoparticles in polymer / biopolymer systems is reviewed at the beginning of Chapter 6.

Figure 2.6: Magnetic Drug Targeting cartoon (Alexiou et al., 2010).
Chapter 3

Magnetic, dimensionally-defined particles: Fabrication, functionalization, and application to magnetofection

3.1 Introduction to shape-defined micro- and nanoparticles

The use of physical properties as a relevant design metric for creating biologically useful micro- and nanoscale particles is a notion which is derived from the elaborate, highly evolved morphologies which are observed in biological systems. Designing particles for specific biomedical applications has the potential to transform the field by creating particles with specifically engineered shapes, sizes, compositions, sur-
face morphologies, optical properties, and mechanical characteristics for optimally performing a specific task. In nature, shape plays a key role in determining how entities move through various media and advanced techniques for manufacturing shape-defined particles may make optimization of particle shape a very realistic parameter capable of being tested \textit{in vitro} prior to \textit{in vivo} experimentation. There is no doubt that shape plays an important role in an extraordinary breadth of physical events, and even more so in the biological world.

Controlling particle composition makes it possible to compound many desired particle properties onto a single particle platform. Specifically, the applications for magnetic particles in medicine and biomedical engineering, such as magnetic resonance imaging, magnetofection, magnetophoresis, and magnetic drug targeting, offer fertile experimental ground for experiments involving magnetic particles. This chapter will describe techniques for (1) making shape defined, compositionally flexible inorganic nanoparticles, (2) assessing the magnetic properties of a subset of these particles using quantitative magnetophoresis, and (3) using these particles as successful magnetofection agents for expedited delivery of a relevant oligonucleotide to cells in vitro. Parts of this chapter are excerpted from a paper published in the Journal of Biomedical Nanotechnology (Mair et al., 2009).
3.2 Previous work on shaping polymeric particles

The basic science and engineering in this field is, at the time of writing, undergoing an explosion of thinking by a diverse body of researchers. Techniques capable of creating particles with unique and finely tuned shapes are being made possible by collaborative efforts between chemists, materials scientists and physicists, as well as electrical, chemical, biomedical, and mechanical engineers. Various research groups have sought to create shape-defined particles, primarily using polymeric materials. Polymeric materials are ideal candidates for the fabrication of shape-defined particles because they are capable of changing phase easily and can be cross-linked using a variety of different techniques.

One simple and efficient technique, pioneered by Samir Mitragotri’s group, involves placing polymeric particles onto a stretchable film, heating the film to within a few degrees of the polymer particle glass transition temperature, and mechanically stretching the film. The mechanical deformations imposed on the film are transferred to the deposited polymeric particles. Using this technique the researchers were able to produce complex particle shapes by inducing complex strains (Champion et al., 2007b; Champion et al., 2007a).

More complex techniques involving lithographic processing of substrates has also resulted in shape-tailored particles. Joseph DeSimone’s group has developed a technique for creating highly shape-defined polymeric and polymer magnetic nanoparticle composite particles by using photolithographically pre-patterned silicon tem-
Figure 3.1: This image shows the particles created by the Mitragotri Group’s method of shape-engineering polymeric particles (Champion et al., 2007a).

plates. In this technique silicon masters are created, surface coated, and filled with the polymer of choice. The polymer fills the wells of the master; crosslinking the polymer results in polymeric particles which have precisely the shape dictated by the silicon master templates. This technique, called Particle Replication in Non-wetting Templates (PRINT), has successfully incorporated a variety of therapeutics, as well as inorganic materials of interest (Euliss et al., 2006; Kelly and DeSimone, 2008; Napier and Desimone, 2007; Rolland et al., 2004).

Also making use of lithographic processes for particle fabrication, Hernandez and Mason created polymeric “LithoParticles” by patterning diversely shaped pillar structures directly onto a wafer using photoresist, developing the UV-exposed resist (crosslinking step), then lifting the newly formed particles off of the substrate. Using this technique they created exclusively polymeric particles in the shapes of letters, showing the high degree of control over particle shape (Hernandez and Mason, 2007).
Figure 3.2: PRINT particle technology developed by the DeSimone research group (Kelly and DeSimone, 2008).
Figure 3.3: Polymeric particles made via lithography and post-liftoff deposition (Hernandez and Mason, 2007).

Significantly, these techniques produce, almost exclusively, polymeric particles, although DeSimone et al. have succeeded in incorporating magnetic nanoparticles into the PRINT particle fabrication platform (Nunes et al., 2010). Creating shape-defined metallic particles makes possible particles with larger magnetic susceptibilities (due to composition, not shape) as well as complex multi-metallic compositions.

3.3 Post-Particles: A novel technique for customized, multimetallic particles

Lithographic techniques for making particles have made significant advances in creating particles with specifically engineered shapes, sizes, and compositions. To
date, the resulting particles have been primarily polymeric particles. Adapting standard lithographic processes, metallic particles were created; the resulting particles have shapes as defined by the lateral dimension of the templates on which they are grown.

Standard photolithography techniques are used to produce 0.20 µm diameter cylindrical posts 0.33 µm tall with 0.70 µm pitch. Briefly, Si wafers (6”) are RCA-cleaned in preparation for photolithography and an ASML 5500/950B Step and Scan photolithography tool (Triangle National Lithography Center, Raleigh, North Carolina) is used to produce post structures. A bottom antireflective coating (BARC) is applied to the wafer prior to photoresist deposition to prevent interfering reflectance from the wafer surface. Following exposure, development and liftoff a metal film is resistively evaporated onto the patterned wafer surface. The resulting film is a laterally conformal layer of metal over the entire wafer surface, leaving only the sidewalls of the vertical post structures exposed to solvents. Iron, iron-gold, nickel-gold-palladium, and nickel-gold-palladium-cobalt particles were created using this method (Figure 3.7). After metallization, templates were placed in acetone for 24h, followed by sonication in acetone for 1h, resulting in the dissolution of photoresist posts and release of evaporated particles (hereafter referred to as Post-Particles). The lateral particle size dimensions were closely defined by the patterned substrate. As shown in Figures 3.4 and 3.5a, the patterned substrate resulted in posts with hemispherical tops. The hemispherical top surfaces of these posts resulted in particles which crescent shaped cross-sections. Figure 3.4 indicates this cross-sectional aspect
of the particle shape. Uniformity in lateral dimension is exhibited by the TEM images in Figure 3.6 in which particles are viewed top-down.

![Patterned substrate M' deposition Pattern dissolution M'' deposition](image)

Figure 3.4: Schematic representation of the bi-metallic particle formation process.

### 3.3.1 Multimetallic particles via Post-Particle methodology

Using these templates particles with varying compositions have been made, including Fe, Fe-Au, Ni-Au-Pd, and Ni-Au-Co-Pd particles. Energy-dispersive X-ray spectroscopy (EDS) analysis was performed on five particle compositions using a Hitachi S-4700 FE-SEM. Three of these particles were Fe-Au particles with varying metal ratios (particles a–c in Figure 3.7), one was a Au-Ni-Pd particle (particle d in Figure 3.7), and the other was a Au-Co-Ni-Pd particle (particle e in Figure 3.7). For compositional analysis, all sample substrates were immersed in acetone (using
Figure 3.5: FE-SEM images detailing wafer surface through all stages of particle fabrication. (a) Lithographically defined posts; (b) metallized wafers result in a post-cap structure; (c) photoresist post dissolution is performed by soaking samples in acetone, leaving a cratered surface with particles and photoresist posts segments in acetone; (d) Au-Ni-Pd particles magnetically concentrated on a substrate. All scale bars are 2 µm.
Figure 3.6: Images taken using a JEOL 2010 TEM. Images indicate spherical component of particle shape as well as size uniformity. The particles are polycrystalline.

a 20 ml scintillation vial) for 24h and sonicated for 1h, after which the growth substrates were removed from the scintillation vial and the particles were rinsed three times using magnetic separation. Subsequently, particles were deposited from acetone onto Ted Pella carbon sample substrates, concentrated under a magnetic field gradient, and imaged immediately after drying. EDS data was collected at 13 kV accelerating voltage and 20 μA beam current. Figure 3.7 indicates the five spectra that were collected along with the atomic compositions of each particle batch created.
Figure 3.7: EDS data of compositionally diverse particles. All particles were created from 99.99% pure metals (Sigma) and evaporated at 1x10^{-6} Torr from tungsten evaporation crucibles. This data represents small subset of material design possible with our post fabrication method. Inset image shows the target compositional profile of each particle type.
3.4 **Biomedical applications of Post-Particles**

Magnetic micro- and nanoparticles with a wide array of chemical and physical properties have been synthesized, characterized, modified, and utilized in materials science, biology and biochemistry (Gupta and Gupta, 2005; Pankhurst et al., 2003). These particles have undergone particularly intense research due to their ability to interact chemically and biologically with a sample while providing the experimenter with a physical means by which to spatially manipulate the particle and its bound payload (Tanase et al., 2005). Attaching therapeutic agents (Salem et al., 2003), DNA (Chorny et al., 2007), or antisense oligonucleotides (Krotz et al., 2003) to magnetic particles for drug delivery applications has proven successful in an array of cell lines. Specifically, researchers have been effective in utilizing magnetic field gradients to increase the interaction probability and, consequently, the transfection efficacy, of various moieties attached to magnetic particles.

Although techniques have been developed to fabricate particles in the size range of 40 nm and below, there is a dearth of strategies for size uniform particles in the range of 20 nm to 1 micron. We present a technique that is capable of defining the lateral shape of the particle down to the resolution of lithography (∼20 nm for electron beam lithography) while precisely controlling the particle composition and structure through sequential deposition of user selected metals or dielectrics. While solution-based techniques are no doubt more scalable (although they rarely result in shape-defined particles), the method of Post-Particle fabrication, as implemented,
is capable of approximately $3.7 \times 10^{10}$ particles / wafer (assuming a 6 in. wafer and center-to-center particle distances of 700 nm).

Previous magnetofection experiments have been performed almost exclusively with commercially available colloidal polymer-iron oxide particles created via wet chemistry techniques (Krotz et al., 2003). Particles grown using photolithographically defined templates have been successfully transfected into cells (Euliss et al., 2006), however more effective delivery of an antisense ODN using these types of particles in conjunction with magnetofection protocols has not yet been reported. The ability to incorporate a variety of metals into a single particle and modulate each metal’s concentration allows user-specific tunability on a single wafer platform, thereby expanding the metallic particle toolbox. Utilizing the various properties of metals also makes possible particles with an array of designed surface chemistries. For example, Fe$_3$O$_4$@Ag particles were recently developed, used as antimicrobial agents, and magnetically reclaimed from water samples (Gong et al., 2007); Park et al. were successful in tuning the catalytic oxidation of CO by changing the composition ratios of Rh-Pt nanoparticles (Park et al., 2008). Also, FePt particles were synthesized and functionalized so as to produce photoswitchable ferromagnetic nanoparticles capable of operating at room temperature (Suda et al., 2007). Optically controlling a particle’s magnetic properties is an important step in realizing high density optical memory (Suda et al., 2007).

Magnetofection aims to increase transfection rate by bringing the relevant gene in rapid and direct contact with a cell membrane, as well as decrease the amount
of vector necessary for a single assay. While this is advantageous for in vitro experiments, the tool’s major potential lies in remote controlled vector targeting in vivo (Plank et al., 2003). Physical vapor deposition into ordered arrays of holes or onto posts has been performed using interferometric lithography for use in memory storage applications (Farhoud et al., 1998) and the magnetic properties of these arrays have been studied (Ross et al., 2002). More recently, lithographically defined templates have been used to create polymeric particles with exquisite control over particle shape and composition (Napier and Desimone, 2007; Kelly and DeSimone, 2008; Hernandez and Mason, 2007; Glangchai et al., 2007). These methods are excellent examples of how patterned substrates offer flexibility in shape and composition which is difficult to produce using strictly chemical methods. However, they differ from our procedure in that they do not rely on metal evaporation onto a surface followed by particle harvesting. We demonstrate the ability to create versatile, size uniform metal particles incorporating from one to four metals in a single particle. We quantified the low-field particle susceptibility and the forces generated on these fabricated particles and compared them with commercially available particles. We demonstrate the usefulness of Fe particles made in this manner by the successful magnetofection of iron/iron oxide particles into HeLa EGFP-654 cells. The transfection successfully delivers antisense oligonucleotide, blocking a mutated, aberrant splice site contained in the EGFP pre-mRNA, resulting in expression of the green fluorescent protein.
3.5 Post-Particles force calibration

To understand the applicability of our particles for magnetofection, we calibrate the force generated on Fe particles created using this technique by an applied magnetic field gradient through the application of Stokes law. Specifically, magnetic fields due to permanent magnets or magnet arrays will play a role in future drug delivery applications of magnetic carriers (Lubbe et al., 1999; Alexiou et al., 2000; Lubbe et al., 2001; Alexiou et al., 2006). In this treatment, quantitative magnetophoresis was chosen over more standard modes of magnetic particle characterization so as to gain an understanding of single-particle force-velocity relationships. Other magnetometry experiments commonly sum over larger volumes of particles and may or may not be good at assessing individual ferromagnetic particles. Quantitative single particle magnetophoresis offers a simple and efficient method for obtaining the desired results.

Particle motion through water was characterized by applying a known field gradient (using a NdFeB permanent magnet) to particles in a closed sample well (20µl). In the low field limit, the magnetic force on a particle is

\[ F = \pi d^3 \frac{\mu_r - 1}{4\mu_0 (\mu_r + 2)} \nabla (B^2) \]  

where \( d \) is the bead diameter, \( \mu_0 \) is the relative permeability of free space (in SI units), and \( \mu_r \) is the relative permeability of the bead (Fisher et al., 2006b). A particle’s
permeability is related to its susceptibility via

\[ \mu_r = \mu / \mu_0 = 1 + \chi_v. \]  

(3.2)

If force \( F \), particle diameter \( d \), and magnetic field \( B \) and field gradient \( \nabla B \) are known, then \( \mu_r \) can be determined using Equation 3.1. Susceptibility can then be calculated using Equation 3.2.

The magnetic field and field gradient were measured to be sufficiently uniform over the 60 \( \mu m \) field of view (Fisher et al., 2006b). Obtaining the solution viscosity (\( \eta \)), bead radius (\( a_b \)), and the bead velocity (\( v \)) allows us to use Stokes formula,

\[ F = 6\pi \eta a_b v \]  

(3.3)

to calculate the average force on each particle. Velocity measurements were also taken on Micromod nanomag®-D PEG-COOH 250 nm particles (Micromod Partikeltechnologie GmbH) and Dynabeads® MyOne™ 1 \( \mu m \) iron oxide impregnated beads (Invitrogen) for comparison. The magnetic field was measured in 250 \( \mu m \) increments along the axis of the magnet; measurements were taken between 18 and 80 mm from the magnet face. A 100x objective was used to image particles (in an epi-illumination configuration) and video was collected at 20 frames per second using a Pulnix PTM-6710CL camera. Video tracking and analysis software (Taylor, 2009) was used to calculate particle velocities under a given field gradient.

We determined the forces on 1 \( \mu m \) superparamagnetic Dynabeads® MyOne™,
250 nm superparamagnetic Micromod nanomag® particles, and 200 nm Fe Post-Particles. MyOne™ and Micromod particles were each diluted 1/10,000 v/v prior to beginning force experiments. Sample volumes were placed in a closed well to eliminate the effects of evaporation and sample drift; the well was spaced a calibrated distance from the face of a permanent magnet. We delivered 20 µl of diluted particle solution (25°C) into the well and observed particle velocities. Particle motion was determined to be at a constant velocity and moving in a straight line with respect to the magnetization axis of the magnet. We identified many-particle aggregates and aligned strings of particles and omitted them from the tracking results. Due to their shape-uniformity, aggregates or aligned strings of two or more MyOne™ beads were clearly evidenced by their lemniscate-shaped image and multi-particle strings were not tracked. The shape heterogeneity of Micromod 250 nm particles makes single particle identification more difficult. MyOne™ beads did not aggregate, while approximately 30% of objects in Post-Particle samples appeared to be incorporated into field-aligned particle chains and 50% of objects in Micromod nanomag® samples were considered aggregates. Although both Micromod and Post-Particles exhibited connected multi-particle groupings during the experiment, these groupings displayed drastically different morphological characteristics within each sample. Micromod particles were observed as both spheres of varying diameter and as field-aligned chains of particles. While aligned strings of Post-Particles were observed, spherically shaped agglomerates of varying diameter were not seen. A reticule was used to determine that the pixel dimension of the video data taken
was 124 nm². Particle velocities were established using in-house particle tracking software (Taylor, 2009). Tracked particles were chosen based on dimensions of the particle image. Figure 3.8 shows the displacement versus time data for the three particles tested.

From the observed particle velocities we calculated the applied forces on the particles due to the magnetic field gradient via Stokes formula. We tracked 45 beads for each type of particle, all tracks occurring in the same magnetic field gradient. Average velocities were 11.1 µm/s, 5.9 µm/s, and 10.7 µm/s for Dynabeads® MyOne™, Micromod 250 nm, and 200 nm Fe Post-Particles, respectively; the resulting average forces applied to these beads were 138±22fN, 18±2fN, and 26±5fN for Dynabeads® MyOne™, Micromod 250 nm, and 200 nm Fe Post-Particles, respectively. Although we have treated the resulting force histogram shown in Figure 3.9 as monomodal for all particle types, the shape of the Post-Particle distribution suggests a bimodal force distribution for Fe Post-Particles, the first mode existing at 22 fN and the second mode occurring at 30 fN. This indicates force data arising from two discrete populations, namely a single particle population and a double particle population. An aligned pair or triplet of particles is expected to experience more force in a given field and field gradient.

We use this force data to calculate susceptibility $\chi_v$ and magnetization $M$ for each particle type. As noted earlier, the force on compositionally equivalent particles which vary in size is proportional to the cube of the particle diameter, $F \sim d^3$. The force applied to a particle in the low magnetic field limit depends on the particle
Figure 3.8: Here we show the magnet response of the particles tested. (a) Blue traces represent 1 µm Dynabeads® MyOne™ superparamagnetic beads; green traces represent Micromod 250 nm particles; purple traces represent 200 nm Fe Post-Particles
Figure 3.9: A histogram representing forces applied to particles. Force calculations are based on Stokes law and are calculated for each bead based on the tracked bead velocity. The inset represents magnetic field measurements taken at 250 µm increments measured using an F.W. Bell model 5080 gaussmeter. A Ni-Cu-Ni coated NdFeB permanent magnet (1 in. diameter, 2 in. length, K&J Magnetics) was used. A surface field of 6775 G was reported by the manufacturer.
volume \( (V) \), the difference in magnetic susceptibilities \( (\Delta \chi) \) (between the particle susceptibility, \( \chi_{\text{particle}} \), and the surrounding material’s susceptibility, \( \chi_{\text{surround}} \)), and the field and field gradient of the magnetic field:

\[
F = \left( \frac{V \cdot \Delta \chi}{2 \mu_0} \right) (B \cdot \nabla) B
\]

Particles we tested varied by size and composition. MyOne™ beads consist of \( \gamma \)-Fe\textsubscript{2}O\textsubscript{3} dispersed in a polystyrene matrix and coated with a thin polymer layer; Micromod particles consist of Fe\textsubscript{3}O\textsubscript{4} dispersed in a silica-fortified polysaccharide matrix (Gruttner and Teller, 1999); 200 nm Fe Post-Particles are iron particles with a native oxide at the particle surface. Using Equation 3.4 and the calculated average forces applied to each type of particle we obtain the volumetric particle susceptibilities. Using the calculated \( \chi_v \) and the known magnetic field of 0.081 T, as well as the relationships among \( B, M, \) and \( H \) [namely, \( \chi_v = M / H \) and \( B = \mu_0(M + H) \)] we determine each particle’s magnetization in the given 0.081 T field. These results are summarized in Table 3.1. The calculated magnetization, \( M \), for the particles is equal to the magnetic moment per unit volume, \( M = m / V \). The magnetization value for Fe Post-Particles was only slightly higher than that of the Micromod particles, while the susceptibilities for Fe Post-Particles was 171% larger than that of Micromod particles.

We now compare our calculations for MyOne™ and Micromod particles with other reports. Previously, researchers have recorded various values for the magnetization and volumetric susceptibility of MyOne™ (Lany et al., 2005; Fonnum
Table 3.1: (a) $\chi_v$ is the volumetric susceptibility and is a measure of how magnetizable a substance can become in a given magnetic field. (b) $M$ is the intensity of magnetization and is defined as the quantity of magnetic moment per unit volume. The data is collected at a field of 0.081 T (see Figure 3.9 inset).

<table>
<thead>
<tr>
<th>Particle</th>
<th>Calculated $\chi_v$</th>
<th>Other reports of $\chi_v$</th>
<th>Calculated magnetization, $M$($\times 10^4$, A/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyOne™</td>
<td>1.4</td>
<td>1.5$^{[32]}$, 1.38$^{[33]}$</td>
<td>3.8</td>
</tr>
<tr>
<td>Micromod</td>
<td>12.6</td>
<td>6±2$^{[34]}$, 4.8$^{[35]}$</td>
<td>6.0</td>
</tr>
<tr>
<td>Post-Particles</td>
<td>34.2</td>
<td>-</td>
<td>6.3</td>
</tr>
</tbody>
</table>

et al., 2005) and Micromod (Ejsing et al., 2004; Freitas et al., 2007) particles. Our measurements of MyOne™ bead susceptibility agrees with these previous reports; our measurements of Micromod bead susceptibility is significantly higher than other reports. This strongly indicates that the Micromod particles tracked and tabulated during our experiment were actually small packets of particles clumped together; our experiments yielding average per particle $\chi_v$ values of 200% and 260% of those previously reported (Ejsing et al., 2004; Freitas et al., 2007) indicates that these small packets typically consisted of two or three particles attached to one another.

Understanding the force-to-size ratio for magnetic particles in a known field and field gradient is crucial for informed estimates of particle translational performance. Importantly, this force-to-size ratio is highly dependent upon the specific material(s) incorporated into a particle. Most particles synthesized via wet chemical methods are composed of magnetite or maghemite (Gupta and Gupta, 2005). As stated previously, for spherical particles of a given material in a given field, a 200 nm particle would be
expected to experience 0.80% and 51% of the force experienced by a 1 \( \mu \)m and 250 nm particle, respectively. Deviations from this pattern must indicate geometric or compositional differences among the particles. Our 200 nm Post-Particles experienced a force 19% that exhibited by 1 \( \mu \)m Dynal beads and 144% that experienced by the Micromod particles, drastically larger than the expected 0.80% and 51%, respectively. Although geometric differences do exist among the particles tested, these differences are too small to cause such large disparities in particle force. These large discrepancies in force mean that our Post-Particles are made from a different material than either of the two commercially available particles. These commercial particles are composed of iron oxide grains smaller than the single domain size (Amblard et al., 1996; Gruttner and Teller, 1999) and, consequently, MyOne\textsuperscript{TM} and Micromod beads are both superparamagnetic (Amblard et al., 1996; Gruttner and Teller, 1999). Collected video data of the absence of aggregation confirmed this fact. In the absence of a magnetic field we observed that MyOne\textsuperscript{TM} - MyOne\textsuperscript{TM} and Micromod - Micromod bead collisions did not result in permanent bead aggregation. This is expected for superparamagnetic particles. In the absence of a magnetic field superparamagnetic beads have no remnant magnetization and therefore no permanent dipole moments. Consequently, there were no dipole-dipole attractive forces between superparamagnetic particles to cause aggregation.

In contrast, aggregation outside of a magnetic field was observed in our Post-Particles. Video data collected in the absence of an applied field showed non-reversible particle agglomeration (the agglomerates were not included in the force
data), indicating that our Post-Particles were ferromagnetic. Ferromagnetic particles, once exposed to a magnetic field, exhibit a net magnetization which exists inside the volume despite the absence of an applied field. The fact that Post-Particles had comparatively large force-to-size ratios (relative to MyOne™ and Micromod particles) in the applied field is due to these differences in magnetic ordering. While MyOne™ and Micromod particles are composed sub-10 nm grains of uniformly oxidized iron (\(\gamma\)-Fe\(_2\)O\(_3\) and Fe\(_3\)O\(_4\)), respectively, our Post-Particle fabrication technique essentially creates arrays of size-uniform metallic clusters with grain sizes significantly larger than 10 nm. These grains are not uniformly oxidized and contain segments of pure Fe; this is not present in either of the superparamagnetic particles. It should be noted that we have used the equation \(\chi_v = M/H\) to calculate magnetization for all particles. This assumption does not hold precisely for ferromagnetic materials. The non-linear hysteresis loop of ferromagnetic materials makes this only an approximation.

### 3.6 Magnetofection using Post-Particles

#### 3.6.1 Attachment of antisense oligonucleotide to Fe particles

Particles may be functionalized while still attached to their posts or after release from the wafer. Polyethyleneimine is commonly used to bind DNA to particles (Boussif et al., 1995; Abdallah et al., 1995; Plank et al., 2003; Gupta et al., 2007; Ragusa et al., 2007) and is used here to electrostatically interact with a EGFP-correcting oligodeoxyribonucleic acid (ODN). Resina et al. have delivered splice-
switching oligonucleotides to HeLa pLuc cells for the successful modulation of a transgene (Resina et al., 2007). These antisense ODN constructs were synthesized using phosphoramidite chemistry (courtesy of Md. Rowshon Alam, University of North Carolina at Chapel Hill, Department of Pharmacology) (Alam et al., 2008). Specifically, the oligomer consists of 20 nucleotides (5’-GTT ATT CTT TAG AAT GGT GC-3’) and is antisense to the cryptic splice site activated on intron 2 of the IVS2-654 mutant beta-globin gene in the HeLa EGFP-654 cells. The ODN is modified with a 2’-O-methyl and phosphorothioate internucleotide backbone, resulting in increased stability and protection against degradation by nucleases.

PEI was bound to the Fe Post-Particles by mixing the particles (1 mg/ml) with 0.1 mg PEI for 4h in a DI water adjusted to pH 10 using 0.1M NaOH. At this pH the zeta potential of the particles is sufficiently low to electrostatically bind cationic PEI to the particle surface. To bind the oligonucleotides, PEI-labeled particles (1 mg/mL) were mixed with oligonucleotide (50 µM) at a 5:1 ratio by volume with an equal part of phosphate buffered saline. This mixture was allowed to rotate for 1 hour at room temperature. For Lipofectamine transfection experiments, antisense oligonucleotide was complexed with Lipofectamine 2000 at a 1:1 volume ratio (Invitrogen, Carlsbad, CA). Lipofectamine 2000 is a common transfection agent used for introducing genes into cells, both transiently and stably.
### 3.6.2 Cell culture and magnetofection experiments

The HeLa cell line EGFP-654 expresses a construct in which the coding region of the of enhanced green fluorescent protein (EGFP) is interrupted by the mutated beta-globin intron. The cell line is grown at 36.5°C and 3.5% CO\(_2\) in Dulbecco’s Modified Eagle Medium with F-12 nutrient mixture (Invitrogen, Carlsbad, CA) with L-glutamine and HEPES buffer supplemented with 10% fetal bovine serum. Growth media was further supplemented with 10% antibiotic-antimycotic (Penicillin-Streptomycin and Amphotericin B, Invitrogen).

HeLa EGFP-654 cells were plated at 5000 cells per well 24 hours before transfection in a Nunc 384 well plate. Before transfection cell media was replaced with Opti-MEM I Reduced-Serum Medium (Invitrogen, Carlsbad, CA). The cells were treated for 1 hour with a 200 nM dose of antisense ODN complexed with 200 nm Fe nanoparticles with and without a permanent magnetic field. Control experiments were performed using untreated and Lipofectamine treated (no particles) HeLa EGFP-654 cells in order to compare the efficacy of gene transfection using these particles. Also, in order to assess the differences between free-floating PEI-ODN complex and particle and Lipofectamine transfections, PEI (50% w/w, 60,000 MW) was diluted 1:5000 and conjugated with the antisense oligonucleotide. It was also added to the cell culture at 200 nM and allowed to incubate for 1 hour, at which time the media was replaced with fresh media.

For magnetic nanoparticle transfections one sample was placed over the NdFeB magnets for 1 hour and a second sample did not receive an external magnetic field.
The media was changed in all samples to D-MEM F-12 with 10% FBS after 1 hour. Replacing media after the field application removed free-floating ODN-laden particles from the solution, as well as any free ODN which may have separated from particles, thus eliminating the possibility for transfection later in the experiment. The cells were incubated for an additional 23 hours before imaging. The total duration of the experiment was 24 hours. After 24 hours fluorescence microscopy was used to quantify the transfection efficacy. Treated and untreated cells were imaged using a Nikon Eclipse TE 2000-E inverted optical microscope (Tokyo, Japan) with a 40x objective. Images were taken using a Photometrics Cascade II 512 electron multiplying CCD digital camera (Roper Scientific, Inc., Tuscon, AZ) and background fluorescence was established by performing segmentation imaging on highest intensity cells in untreated samples. Cells measuring above this background fluorescence in treated samples were measured for mean intensity using segmentation. Mean background fluorescence was subtracted to determine the true mean intensities above background for treated cells. An average of 20 images were taken of each well and weighted mean intensities were summed over all images.

3.6.3 Magnetically enhanced cell transfection

The general mechanism of the magnetic transfection starts with introducing the DNA-particle complexes into the cell media. A magnetic field is applied to direct the particles to the cell membrane. The particles come in contact with the cell membrane and are eventually taken into the cytoplasm via endocytosis. The particle
becomes encapsulated by a membrane-bound endosome as it is moved into the cellular interior. The PEI polymer has the capability to disrupt this membrane through a process called endosomolysis (Huth et al., 2004). The PEI seems to elicit a substantial proton influx into the endosome ensued by a passive chloride influx. This causes osmotic swelling of the endosome, causing its rupture, and the subsequent release of the particle and genetic products. From here, a certain portion of the DNA is released from the particle to perform its designed function.

Although lithographic techniques have gained popularity as a means of tailoring particle shape and composition (Rolland et al., 2004; Hernandez and Mason, 2007; Napier and Desimone, 2007; Kelly and DeSimone, 2008), increased transfection via magnetofection using these types of particles has yet to be reported. In order to test the comparative efficacy of EGFP turn-on via particle delivery of ODN, five sets of cell treatments were established: untreated cells, cells dosed with only the PEI-ODN complex, cells dosed with antisense ODN-bound 200 nm Fe Post-Particles delivered without a magnetic field gradient, antisense ODN-bound 200 nm Fe Post-Particles delivered to cells in the presence of a field gradient, and cells treated with Lipofectamine 2000. Figure 3.10 shows representative images from each treatment course. The efficacy of magnetofection using our Post-Particles is clearly evidenced by the increased expression of EGFP. The increased fluorescence is a product of particles penetrating the cell membrane, delivering antisense ODNs to the cell nucleus, and aberrant splicing of EGFP pre-mRNA being corrected. Results from segmentation analysis of EGFP-expressing cells indicate a 76% increase in fluorescence intensity
of ODN-loaded magnetic particles (with magnetic gradient applied) over Lipofectamine as well as a 139% increase in intensity over the non-magnetic control (Figure 3.11). Also, as evidenced by images taken throughout the well, the rate of transfection was significantly higher in the magnetic sample over the non-magnetic control.

Magnetofection experiments exhibited only low levels of cell death (less than 5% overall) as observed through bright field microscopy and no evidence was seen that our particles increased cytotoxicity. However, our transfection times were 1 hour, and as such, possibly not a long enough time frame to accurately gauge toxicity levels. Toxicity experiments performed over extended periods would provide greater information regarding the comparative toxicity of our particles. Likewise, untreated HeLa EGFP-654 cells were unresponsive to the applied magnetic field and showed no visible signs of increased cytotoxicity during the experiment. It has been noted previously that an applied magnetic field enhances the transfection rate. The general mechanism by which this increased endocytosis is not clear (McBain et al., 2007). It is accepted that the process of magnetofection decreases the time necessary for cell-particle interactions to occur by magnetically placing the ODN-laden particles spatially near or on the cell membrane. Researchers have explored the application of oscillating fields (McBain et al., 2007; Kamau et al., 2006) and have speculated that the oscillating field increases efficiency of endocytosis and may also cause endosomal release.
Figure 3.10: Representative images of (a) untreated cells, (b) PEI-ODN treated cells (c) cells treated with particles labeled with oligonucleotide (200 nM) and delivered to cells without an applied magnetic field; (d) cells treated with particles labeled with oligonucleotide (200 nM) and delivered to cells with an applied magnetic field; (e) Lipofectamine treated cells.
Figure 3.11: Average fluorescence intensities based on segmentation data collected across wells.

3.6.4 Conclusions for Post-Particle biomedical applications

Multimetallic particle synthesis via physical vapor deposition onto lithographically defined substrates offers a method by which to produce highly size and shape uniform particles. Our technique allows user-specific metal compositions and concentrations, making this technique useful for tailoring the particle properties to a high degree. Particle velocities resulting from the application of a known magnetic field gradient were used to calculate the average force applied to particles and compare this with average forces applied to commercially available particles in the same field gradient. Particle susceptibilities were calculated and compared with expected values based on the particle composition. The significant forces applied to these nanoscale particles in the applied field gradient makes them excellent candidates
for biophysical force experiments where small size is preferred (such as intracellular environments), and where large force is also a requirement. Finally, results represent the first time lithographically-templated particles have been used in conjunction with magnetofection to deliver genes to cells.
Chapter 4

Templated Electrodeposition of Micro/Nanorods

In this chapter I discuss experiments based on nanorods fabricated via templated electrodeposition. I briefly discuss the basics of the technique and detail the fabrication of a variety of different nanorod and nanowire materials (both simple, single-component nanorods and axially multisegmented nanorods) using the technique. After this introduction, I detail the fabrication of Janus-structured magnetic nanorods (a class of nanomaterial which, to date, has not been reported) based on electrodeposited Au nanorods. Finally, this chapter uses these magnetic Janus nanorods as rotational near-surface swimmers capable of directed translation in the two-dimensional plane microns above a glass slide. This chapter details experiments using these magnetic Janus nanorods for microbead capture and manipulation, as well as directed single cell translation. Parts of this chapter are excerpted, with permission, from a paper in the Journal of Physics D: Applied Physics (Mair et al.,
4.1 Templated electrodeposition for fabricating magnetic rods and wires

Dimension-controlled particles can also be created using the technique of template electrodeposition. As early as 1970 Possin succeeded in selectively etching the damage tracks produced by bombarding mica with high energy ions (Possin, 1970). The result was a flat surface with 15 \( \mu \text{m} \) deep, 40 nm diameter pores. Electroplating into the pores of this material resulted in the first demonstration of template-guided electrodeposition for the formation of nanowires. This general scheme for nanowire synthesis did not enjoy widespread use until it was adapted by R. M. Penner and C. R. Martin in the late 1980s and early 1990s; these authors made use of microporous polycarbonate host membranes as opposed to etched mica, and are generally considered to have pioneered the field (Penner and Martin, 1987; Martin et al., 1990; Martin, 1991; Martin, 1994; Martin, 1995). Generically, electrochemical deposition (or electrodeposition, or electroplating) relies on the reduction of metallic ions (solution) onto a conducting electrode at the base of the template pores, the template typically being tens to hundreds of micrometers in thickness. For the nanorod materials grown in this chapter, all deposition solutions are aqueous, consisting of a metal salt, boric acid, and other additives (see Appendix A). The general chemical reaction for converting metal ions to solid material is
The process is most commonly employed using polycarbonate track etched (PCTE) membranes or anodized aluminum oxide (AAO) templates. A few important differences exist between the two host membrane materials. Specifically,

- PCTE membranes are polymeric and typically rather flexible, AAO membranes are ceramic and brittle;
- PCTE membranes have randomly arranged pores, while AAO membranes have hexagonally ordered pores;
- PCTE membranes melt at temperatures above 60°C, AAO membranes can withstand temperatures on the order of 400°C;
- PCTE membranes are susceptible to dissolving in organics such as dichloromethane, while AAO membranes are resistant to common organics, but are etched using NaOH or KOH.

Regardless of which template type is used, fabrication of rods and wires requires closing off one end of the template’s pores; in our case this is done with a layer of thermally evaporated Ag. An important methodological note when backing these templates via thermal evaporation is that the template should be held at a slight angle with respect to the source. Thermal evaporation is a line of sight deposition process, and this angle ensures that the evaporated metal does not coat the interior walls of the
membrane pores. The resulting micro- and nanomaterials are dimension-defined in that their diameters are determined by the template pore diameters, and the length of the resulting segment is a function of the amount of charge (measured in mC) deposited into the membrane.

As compared to solution routes to nanowire synthesis, templated synthesis offers a simple method for tightly controlling wire dimensions; it is capable of creating conducting (Yu et al., 1997), semiconducting (Pena et al., 2002; Law et al., 2004), polymeric NWs (Ramanathan et al., 2004), as well as carbon nanotubes (Hwang et al., 2005). Additionally, the template walls can be actively used to manipulate spatially discrete regions of chemical functionalization along the NW sidewall (Skinner et al., 2006).

In this chapter all NWs will be produced using AAO templates. These templates are purchasable in a wide variety of thicknesses and pore diameters, and it is possible to obtain commercial templates with pore diameters ranging from five nanometers up to several hundred nanometers. The wires shown in this chapter are derived exclusively from either Whatman, Inc. Anodisc AAO (Maidstone, Kent, UK) or Synkera Technologies, Inc. AAO (Longmont, Colorado, USA). Templates are 50 µm thick, and Synkera templates with 18, 55, and 100 nm diameter pores were used to grow wires. These templates have pore densities of $5 \times 10^{10}$, $5 \times 10^{9}$, and $2 \times 10^{9}$ pores/cm$^2$, respectively. Whatman Anodisc templates have 200-300 nm pores, with pore densities of $9 \times 10^{8}$ pores/cm$^2$. For readers using this document as a guide to template-guided rod synthesis, a few important technical notes on the preparation
and use of templates with various pore dimensions can be found in Appendix A.

4.1.1 Multicomponent rods - multisegmented rods and wires

The Post-Particles discussed in Chapter 3 were capable of being multimetallic because several different metals could be thermally evaporated onto a single templated surface. In a similar manner, electrodeposited rods can be made multicompositional by simple sequential electrodeposition. Researchers have extensively made use of the templated electrodeposition method for making nanowires to create multisegmented rods and wires (Hurst et al., 2006). While many researchers have used the multisegmented methodology for adding optical, magnetic, chemical, or mechanical functionality to the rod length, this section of the document utilizes the multisegmented technique purely as a means of creating more particles per template. Depending on the materials involved in the deposition process, multilayered nanorod synthesis can be performed by either (1) changing the electrolyte in the chamber, or (2) changing the applied potential while using an electrolyte containing two metal ions, each of which is deposited at a different potential. For experiments performed in Chapter 6 I use the multilayered deposition of Cu and Ni (alternately), deposited from a single electrolyte solution. The Cu segments of the resulting Cu-Ni multisegmented wires are then etched in 5M KOH, leaving only the (now separated) ferromagnetic Ni segments behind. Fabrication details can be found in Appendix A.
Figure 4.1: Electrodeposited Cu-Ni rods grown from a single electrolyte. The more rough segment is Cu, while the more smooth segments is Ni.

Figure 4.2: A composite of the various dimensions of rods / particles grown via the electrodeposition technique.

### 4.2 Fabricating Janus nanomaterials

High aspect ratio magnetic micro- and nanoscale wires and coils and have proven effective in biomanipulation (Ghosh and Fischer, 2009), tissue penetration (Ishiyama et al., 2001), and drug delivery (Salem et al., 2003; Pearce et al., 2007). Template-grown magnetic nanowires have successfully manipulated large arrays of multicellular constructs (Tanase et al., 2005) and single cells (Hultgren et al., 2003; Zeng et al., 2009), even succeeding in the positioning of a single neuron across a microfabricated electrode (Choi et al., 2007). These magnetically driven devices are unique in their
ability to operate wirelessly, without attached power supplies or on-board controlling appliances, and in a variety of materials and fluidic settings. These qualities make magnetically driven micro-swimmers and micro-rotors potential candidates for biomedical applications in cell manipulation, microfluidics, and nanorheology.

A common attribute of these micro- and nanosized devices is an engineered asymmetry of the particle surface. These anisotropic Janus particles are unique in two respects. As shown recently by Ghosh et al., having magnetic material exclusively on one side of a corkscrew shaped nanoparticle allows field-induced particle rotation around the particle axis (Ghosh and Fischer, 2009). Additionally, Janus particles expose multiple materials to the surrounding environment, allowing for potentially diverse chemical, electrical, optical, or magnetic properties to be incorporated onto single-particle platforms (Shah et al., 2009; Walther et al., 2009; Roh et al., 2005; Lattuada and Hatton, 2007).

Previous researchers have successfully used template-directed electrodeposition to create nanowires with a diverse array of compositions along the wire axis (Attenborough et al., 1995; Wang et al., 1996; Wildt et al., 2006; Elnathan et al., 2008). Additionally, Qin et al. developed a technique for modifying wires along the wire radius for single-molecule electrical measurements (Qin et al., 2005; Martin and Baker, 2005), catalytically driven nanorotors (Qin et al., 2007b), and nanodisc barcodes (Qin et al., 2007a); Chen et al. recently used this technique for fabricating rod-sheath heterostructures capable of focusing plasmons (Chen et al., 2009).

Expanding the nanowire toolbox, we fabricate magnetic Janus nanowires via tem-
plated electrodeposition and post-growth thermal evaporation of magnetic layers. We then use rotating magnetic fields to induce rotational, and consequently translational, motion in these Janus nanowires. We show (1) that increasing the number of rotations per minute is correlated with an increase in translational velocities and (2) that by changing the position of a bar magnet located above the solution-dispersed Janus wires we are able to successfully manipulate our wires along the X and Y axes of a sample substrate. Additionally, we incubate magnetic Janus wires with human bronchial epithelial cells and successfully manipulate these cells via rotational maneuvering using both Janus wire agglomerates and single wires. This work represents the first demonstration of magnetically responsive Janus nanowires and their application to single cell manipulation.

### 4.2.1 Fabrication of magnetic Janus rods

Template-based techniques for fabricating nanorods, nanowires and nanotubes are simple, efficient, and inexpensive and have been described by a several other researchers (Hangarter and Myung, 2005; Hurst et al., 2006; Hangarter et al., 2007). Briefly, electrodeposition of Au wires was performed into the pores of commercially available Whatman AAO templates with nominal pore diameters of 200 nm. Templates are prepared first by thermal evaporation of a Ag working electrode (450 nm thickness) onto one side of the template. Following thermal evaporation, the templates are placed in a custom-made electrodeposition cell; deposition is performed using a standard three-electrode setup (Pt auxiliary electrode, Ag/AgCl reference
electrode) and a commercially available Au electrolyte (Technic Inc., Orotemp 24 RTU Rack). We grow Au wires approximately 5.5 µm to create our Janus NWs, however the technique we present is amenable to any (inorganic) wire composition. Following deposition the Ag working electrode is etched in HNO₃ and the wires are released from the template via AAO dissolution in 1M NaOH. Wires are then rinsed 5 times in ethanol via gentle centrifugation and sonication, and redispersal in ethanol. Following rinsing wires are diluted to approximately 2.5x10⁷ wires/ml and deposited onto Cr-coated Si substrates (based on calculations of template pore density and deposition surface area). Cr is a commonly used adhesion layer between various metals and SiO₂ (Fennimore et al., 2003; Papadakis et al., 2004; Wei et al., 2008). In our experiments the Cr layer eliminates unwanted removal of Ni-Cu material from the substrate during subsequent sample sonication. Following wire deposition, these substrates are coated with a 50 nm Ni film and a 15 nm Cu film via thermal evaporation. Ni-Cu layers are used due to the high susceptibility of thermally evaporated Ni layers and their resistance to chemical dissolution; Ni-Pd layers were also used with equal success. Significantly, this technique, is amenable to engineering these layers for specific purposes with respect to material and layer thickness. Subsequent sonication in isopropanol (IPA) (10 seconds sonication time, 12 W sonicator) results in Au wires coated (along one side) with Ni–Cu Janus layers suspended in solution; the substrate is removed from the solution after sonication and the Janus wires are rinsed five times in IPA. Scanning electron microscopy of the substrate surface after a partial removal of Janus nanorods is shown in Figure 4.3b.
Voids left in the Ni-Cu thin film indicate the positions of nanorods that have been removed into solution, each rod now possessing a partial magnetic coating. Direct transmission electron microscopy confirms the presence of the deposited layer on individual rods compared to untreated Au material (Figure 4.3c & d). Approximately 60% of each nanorod’s surface area (excluding rod faces) is covered by the deposited Ni-Cu or Ni-Pd layers.

4.2.2 Characterization of magnetic Janus wires

The described technique yields a novel form of magnetic Janus nanowires. Figure 4.3b shows an SEM image of a sample substrate after application of a Cr adhesion layer, initial NW deposition, NW Janus coating deposition, and partial removal of newly Janus NWs via sonication are performed. Importantly, this process is highly material independent and can be used to create multi-surfaced NWs with an extensive combination of NW and Janus layer materials. TEM images of our wires indicate that this process results in wires which have 60% of their outer perimeters covered by the evaporated Ni-Cu or Ni-Pd layers.
4.3 Manipulating Janus rods near a surface

4.3.1 Introduction to payload capture and manipulation at the micrometer scale

Biological motors are amazing, capable of performing specific tasks with incredible efficiency. Motor proteins shuttle vesicles, organelles, and other cargo around cells, using energy garnered from the hydrolysis of adenosine triphosphate (ATP) to power their motion. Naturally, the field of nanoscience has set out to engineer human-made nanomachines capable of directed guidance, cargo transport, and force application in a variety of settings and making use of a variety of different fuels and propulsion mechanisms. Specifically, micro- and nanoscaled objects capable of being operated wirelessly and in a variety of low Reynolds number (low-Re) solutions hold potential for manipulating and assembling objects at the nanoscale (Ozin et al., 2005; Ebbens and Howse, 2010; Fischer and Ghosh, 2011). At the time of writing, these nanomachines are undergoing intense research efforts, as their applications to nanomedicine, environmental sensing, nanoscale transport, directed assembly / nanorobotics hint at their enormous potential for interacting with systems (both organic and inorganic) at the nanoscale (Wang, 2009). Within this field the nanomachines can be broadly grouped according to their method of transport.

**Catalytic motors:** Chemical-converting nanomachines capable of catalyzing the hydrolysis of H$_2$O$_2$ for propulsion are relatively simple to fabricate, as they can be grown via templated electrodeposition (Sundararajan et al., 2010), as well as several
other methods for localizing Pt on a particle surface (Valadares et al., 2010). Generally, the addition of Pt to a H₂O₂-containing solution results in catalysis of the reaction \( \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \). Frequently, these nanomotors consist of Au-Pt or Ni-Pt nanorods. While the exact propulsion mechanism at work in these metal-Pt systems is still under debate, the generally accepted electrokinetic self-electrophoresis notion suggests that, in addition to the catalysis of \( \text{H}_2\text{O}_2 \) into \( \text{O}_2, \text{H}^+, \text{and e}^- \) at the Pt surface, a secondary reaction takes place on the metal surface. This second reaction results in the reduction of 4 electrons near that surface, causing a general electron flow in the direction of the metal. The general electron motion away from the Pt-end and towards the Au-end results in an equivalent \( \text{H}^+ \) flow in the same direction, resulting in nanorod propulsion in the opposite direction (Wang, 2009). Other mechanisms include propulsion via bubble formation (Fournier-Bidoz et al., 2005), an interfacial tension mechanism (Paxton et al., 2004), and a Brownian rachet mechanism based on local changes in viscosity (Dhar et al., 2006). Motors based on this propulsion method have shown promise for achieving controllable maneuvering (Burdick et al., 2008), high velocities (on the order of 100 \( \mu \text{m/s} \)) (Demirok et al., 2008), and cargo transport and drop-off (Sundararajan et al., 2008; Sundararajan et al., 2010). Of course, one limitation for these nanomachines is their dependence upon chemical fuel (H₂O₂) for propulsion.

**Helical corkscrew propellors:** The helical shape of a corkscrew lends itself well to the fabrication and manipulation of microscale devices, as rotation around the long axis of the corkscrew induces motion. Helical swimmers on the scale of tens
of microns (Zhang et al., 2009) all the way down to one micron (Ghosh and Fischer, 2009) have been fabricated and actuated using this technique. For magnetic actuation, helices are created such that some component of the structure is magnetic – Zhang et al. made use of a magnetic head, while Ghosh et al. used glancing angle deposition to coat one side of their helix structures with a magnetic film. These structures are easy to manipulate and have demonstrated impressive cargo manipulations in solution (Ghosh and Fischer, 2009), however they typically rely on elaborate fabrication schemes.

Near-surface hydrodynamic swimmers: Of particular interest are magnetically driven devices capable of being guided in a variety of materials and fluidic settings by externally applied magnetic fields (Baudry et al., 2005; Ghosh and Fischer, 2009), as these micro-objects can perform complex maneuvers without carrying any onboard fuel. Such devices are valuable for their utilization in bead (Ghosh and Fischer, 2009) and cell (Sakar et al., 2010) manipulation, as well as in-channel microfluidic navigation (Tierno et al., 2008b). Specifically, swimming in a low Reynolds number environment requires a mechanism which breaks the symmetry of the swimmer’s motion (Purcell, 1977; Childress, 1981). Many different types of swimmers have been proposed and demonstrated, such as three-bead swimmers (Purcell, 1977; Najafi and Golestanian, 2004; Becker et al., 2003), linked-bead swimmers (Baudry et al., 2005), flexible magnetic filaments (Keaveny and Maxey, 2008; Belovs and Cebers, 2009), and membranes (Najafi and Golestanian, 2005). Most recently, assemblies of micron-sized magnetic beads have been rotated near a wall in an end-over-end fashion;
the hydrodynamic conditions of the sphere near the wall breaks the symmetry of the rotation and results in a net translation of the micro-object (Tierno et al., 2008a; Tierno et al., 2008b; Tierno et al., 2010; Sing et al., 2010). To date, these reports utilize assemblies of micron-scaled beads or nanorods (Zhang et al., 2010) rotating in a plane nearly perpendicular to the floor.

A note on the Reynolds number for these swimmers: Reynolds number for a swimmer is calculated by considering the swimmer radius $a$, swimmer velocity $v$, fluid density $\rho$, and fluid viscosity $\eta$: $R_{\text{number}} = av\rho/\eta$. Using $\rho/\eta = \nu$, the kinematic viscosity, we get $R_{\text{number}} = av/\nu$. Our swimmers are approximately 5.5 $\mu$m long (so, we’ll make $a = 2.75 \mu m$), swim at approximately 4 $\mu$m/s, and operate in water ($\nu = 10^{-2} \text{cm}^2/\text{s}$), resulting in approximately $R_{\text{number}} = 1.1 \times 10^{-6}$.

Magnetic Janus nanorod swimmers: In this work we present a simple, efficient method for the fabrication and manipulation of rod-shaped nanoscaled swimmers which operate via rotation in a plane nearly parallel to the floor. These swimmers are controlled by a rotating magnetic field and can be manipulated with sub-micrometer precision. While magnetic field gradients can be used to apply forces via passive translation (motion is exclusively in-plane translation), rotational manipulation is an active mode of translation during which rotation around some object axis produces in-plane translation. We rotate magnetic Janus nanorods (possessing two different face surfaces on a single nanorod) located near a glass substrate in a low Reynolds number solution, which we find induces controllable, high spatial resolution in-plane translation. Our model for translation incorporates symmetry breaking
through increased drag at the no-slip surface boundary, and we show that nanorod translational velocity is linearly proportional to angular velocity. Additionally, we demonstrate that orthogonal magnetic fields control in-plane motion arbitrarily, and consequently the rods can be manipulated independently along the $x$- and $y$-axes of a sample substrate by simple changes in the configuration of the applied magnetic field. A small tilt out-of-plane results in symmetry-breaking, as the end of the rod which sweeps closer to the floor experiences an enhanced drag coefficient relative to the other half of the rod. The result is a net translation approximately within the plane of the floor; we call this style of near-surface planar motion “crawling.” In this unique mode, translation speed and direction can be varied significantly by small changes in the angular velocity and direction of the applied magnetic field, respectively. Results hold potential for directed transport applications.

We implement this method of translation using magnetic Janus nanorods previously discussed. Although solid magnetic rods will exhibit similar crawling behavior, these magnetic Janus rods enable the additional benefit of regio-specific chemical functionalization due to the possibility of having, on a single nanorod, two or more exposed metals for surface functionalization. In order to utilize the Janus properties of these rods, we selectively functionalize exposed Au regions with dangling thiol bonds to capture Au-Pd-coated 1 µm polystyrene spheres. Additionally, we incubate magnetic Janus rods with human bronchial epithelial cells (HBE-16) and successfully manipulate these cells via rotational maneuvering using individual rods. This work represents the first demonstration of magnetically responsive Janus nanorods, imple-
ments these rods as a novel form of near-surface nano-swimmer capable of payload manipulation, and models the swimming action mechanism using resistive force theory.

In order to collect video data of Janus nanorod manipulation, we dilute \( \sim 1 \times 10^7 \) rods in 20ml of 1:1 deionized water and isopropanol. Cylindrical sample chambers are fabricated from polydimethylsiloxane (PDMS) sealed to a glass slide. For magnetic manipulation, we deposit rods into PDMS sample wells and place cover glass on top of the sample chamber (and sealed with vacuum grease) to minimize fluid motion due to evaporation. We observe Janus rod manipulations using an inverted transmission optical microscope with a 50x air objective (Nikon). Video data is collected at 60 or 120 frames per second using a Pulnix PTM-6710CL camera and in-house video capture software (a schematic of the microscope setup is shown in Figure 4.4).

Magnetic Janus nanorods are actuated via a NdFeB rectangular magnet (K&J Magnetics, Jamison, Pennsylvania). This magnet is attached to a Barber-Colman inline gear motor driven by a constant external voltage source and positioned above the sample plane (see Figure 4.4). The magnet is positioned such that the field strength at the sample plane is \( \sim 30 \) G and the field direction is nearly parallel to the sample floor. The magnet is then rotated such that the field at the sample rotates in a plane nearly parallel to the floor, but tilted by some small angle \( \theta \). Motor drive voltage is varied to control the angular velocity of the magnetic Janus rods. Video tracking is performed via in-house software (Taylor, 2009) to determine both angular
Figure 4.3: (a) Schematic of rod processing, showing a Cr-coated Si wafer, Au nanorod deposition onto the surface, Ni-Pd film deposition, and Janus nanorod removal via sonication. (b) Partially removed Janus nanorods, showing a subset still adhered to the substrate surface (green arrow), while some rods have been lifted off during the sonication process (red arrow). TEM images of Au rod prior to (c) and after (d) thermally evaporated Janus layer (blue arrow). Cartoon representations are shown to the right.
and translational velocity. The magnet and rotating Janus rods have identical angular velocities, as evidenced by analysis of video data.

We focus exclusively on rods at the bottom glass slide, as translation via rod rotation relies on surface effects. We observe two distinct modes of transport in this way. A less stable, less frequently observed mode of locomotion relies on repeated, end-over-end rod tip friction interactions with the glass surface. During these interactions, direct contact between the rod tip and the glass substrate results in frictional forces which fix the tip at a specific substrate location. The field-aligning behavior of the rod drives the bulk of the rod to pivot around the tip-substrate pivot point. For our magnetic Janus nanorods this “walking” behavior (Figure 4.5d-f) is frequently short-lived (~2 – 4 rod pivots, which we define as “steps”) and is terminated by irreversible adhesion to the glass surface. Single steps of this walking motion occur at discrete increments of nanorod length. Consequently, this mode of translation offers only low spatial resolution for nanorod positioning, with location being controllable by integer values of rod length. Detailed discussions of walking motion can be found elsewhere (Morimoto et al., 2008; Zhao and Zeng, 2009; Zeng et al., 2009). More commonly we observe “crawling” motion (Figure 4.5a-c) which is not dominated by tip-surface interactions, but propels the nanorod in incremental steps much smaller than the rod length. Like the walking mechanism, crawling also relies on near-surface friction in that the increased drag in the no-slip boundary near the substrate surface is directly responsible for the rod propulsion as it rotates. Because nanorod crawling relies on fluid-mediated frictional forces
between the rod and the surface (as opposed to direct contact), the translational motion is incremental and has an individual crawl length (the distance traveled in one rod rotation) only a fraction of the rod length. These nanorod crawlers remain free for tens of minutes without undergoing irreversible rod-substrate interactions. Here, we focus exclusively on the observed crawling motion, as this implementation of the mechanism has not been described previously and, as we will show, allows precise control over lateral translation.

Figure 4.4: Rotation control setup.
Figure 4.5: Two types of rod motion are observed. Crawling (a-c) and walking (d-f). Side view oriented cartoon representations (a, d) indicate no-contact crawling vs. end-over-end contact walking. Microscope images taken from videos of crawling (b) and walking (e). The red triangle tracks the motion of a specific rod tip in the image sequence. Crosshairs have been added as points of reference. Minimum intensity projections (c, f) show crawling and walking, respectively. Scale bar is 10 µm and refers to b, c, e, and f.
4.3.2 Controlling crawling motion

High resolution, variable speed, multidirectional control is compulsory for low Reynolds number swimming devices to be useful in microfluidic, biomedical, and nanoscale assembly applications. We find that the translational velocity due to crawling varies directly with the angular velocity of the nanorod (Figure 4.8). This translational motion is in the direction of $\omega \times z$, where $z$ represents the vertical axis. This translational motion is unidirectional (for a given rotation direction of the drive magnet) and oriented orthogonal to the angle $\theta$ of the nanorod tilt relative to the substrate. Reversal of the magnetic field rotational direction consequently reverses the rotational direction of the Janus nanorods (clockwise or counterclockwise), allowing control over translational direction ($+x$ or $-x$). Additionally, by changing the orientation (and therefore the orthogonal direction) of the magnet we are able to induce translation in any arbitrary direction. In order to demonstrate this control of motion, we manipulate a single nanorod along the path of the letters “u-n-c” (Figure 4.6). Importantly, manipulation by this new crawling mechanism makes it possible to control rod position and velocity in the lateral plane with a resolution of only a fraction of the rod length, as opposed to the walking mechanism, for which the translation resolution is limited to increments of rod length (Zhao and Zeng, 2009; Zeng et al., 2009).
Figure 4.6: Minimum intensity projection of a single nanorod (crawling) manipulation, spelling out the letters “u”, “n” and “c”. Scale bars represent 10 µm.
4.3.3 Model of crawling mechanism

In the current study, symmetry is broken by a combination of nanorod tilt induced by an off-axis magnetic field and the boundary effect of the lower glass surface (floor). We see nanorods undergoing crawling translation near the floor of the sample chamber, where the no-slip boundary condition of the floor results in an enhanced drag coefficient. Since the drag coefficient is larger near the floor, the tilted rotation of the rod results in frictional asymmetry and thus a net translation. Here, we use resistive force theory (Gray and Hancock, 1955) to explain this motion.

A ferromagnetic rod driven by a rotating magnetic field in a viscous fluid will rotate at the same angular velocity as the magnetic field as long as the angular velocity of the applied field, \( \omega \) (rad/s), is less than the critical velocity of the rod-fluid system, \( \omega_c = \frac{\xi}{\gamma} \), where \( \xi \) is the magnetic torque on the rod and \( \gamma \) is the drag coefficient of the rod (Urena et al., 2009). For our system, \( \omega_c \approx 10^4 \) rad/s, which is much greater than typical values of \( \omega \) in this work (typically 2 - 50 rad/s), and so we can assume that \( \omega_{magnet} = \omega_{rod} \equiv \omega \).

The net velocity of a segment of the rod a distance \( \rho \) from its center can be described as the vector sum of its translational and rotational velocities (inset, Figure 4.7a). Decomposing this velocity \( \nu \) into components both parallel and perpendicular to the long axis of the rotating rod, we find

\[
\nu_z = \omega \rho + \nu_t \cos(\omega t), \quad \nu_{\parallel} = \nu_t \sin(\omega t)
\]

\( (4.2) \)
where $\omega$ is the rod angular velocity, $v_t$ is the rod translational velocity, and $t$ is time. We also assume that the angle between the plane of rotation and the horizontal, $\theta$, is small, as determined by observation of apparent rod length during a rotation. The differential drag force on a segment of the rod a distance $\rho$ from the center is then given by

$$dF_\parallel = \gamma_\parallel v_\parallel d\rho; \quad dF_\perp = \gamma_\perp v_\perp d\rho$$

where $\gamma_\perp$ and $\gamma_\parallel$ are the drag coefficients in the perpendicular and parallel directions, respectively. Due to symmetry considerations, there is no net translation in the $y$ and $z$ directions. In the $x$ direction, the net force is given by the sum of the $x$-components of $dF_\perp$ and $dF_\parallel$ as the rod rotates at constant angular velocity

$$dF_x = dF_\perp \cos(\omega t) + dF_\parallel \sin(\omega t).$$

In this model we make use of the drag coefficients given by Hunt et al., (Hunt et al., 1994)

$$dF_x = \frac{2\pi \eta}{\cosh^{-1}\left(\frac{h(\rho, t)}{r}\right)} \left[2\omega \rho \cos(\omega t) + v_t(1 + \cos^2(\omega t))\right] d\rho$$

where $\eta$ is the viscosity of the fluid, $r$ is the radius of the rod, and $h$ is the height of each element of the rod above the floor, which is a function of both $\rho$ and $t$. The height of the rod as it rotates can be expressed by $h(\rho, t) = h_0 + \rho \sin \theta \cos(\omega t)$, where
$h_0$ is the height of the center of the rod and $\theta$ is the angle by which the plane of rotation is tilted from the horizontal. Since the rod rotates in a low-Re fluid, inertial forces are negligible and the net force on the swimmer in the $x$-direction is zero at all times. Thus,

$$F_x = 0 = \int \frac{2\pi \eta [2\omega \rho \cos(\omega t) + \nu_t (1 + \cos^2(\omega t))]}{\cosh^{-1}[(1/r)(h_0 + \rho \sin \theta \cos(\omega t))] - d\rho}$$  \hspace{1cm} (4.6)

Integration yields an expression for the translational velocity of the rod as a function of time, $\nu_t(t)$:

$$\nu(t) = \frac{2\omega \csc \theta}{3 + \cos(2\omega t)} \left( \frac{2\nu_t(\cosh^{-1}A) - r \nu_t(2\cosh^{-1}A) - 2h \nu_t(\cosh^{-1}B) + r \nu_t(2\cosh^{-1}B)}{\nu_t(\cosh^{-1}A) - \nu_t(\cosh^{-1}B)} \right)$$  \hspace{1cm} (4.7)

where Shi($x$) is the hyperbolic sine integral and

$$A = \frac{2h - L \cos(\omega t) \sin \theta}{2r}, \quad B = \frac{2h + L \cos(\omega t) \sin \theta}{2r}.$$ \hspace{1cm} (4.8)

The average translational velocity can be determined by numerical integration over a quarter-period; however, since $\nu_t(t)$ is very nearly sinusoidal, $\nu_{avg} \sim \nu_{max}/2 = \nu_t(0)/2$ yields a good approximation. The average translational velocity is independent of viscosity, as expected for a low-Re swimmer, and increases linearly with angular velocity, as observed in our experiments.
Figure 4.7: Top (a) and side (b) views of the rotating nanorod. The rod rotates clockwise with constant angular velocity, $\omega$, and moves in the $x$-direction with a translational velocity $v(t)$ (a, inset). A point on the rod an arbitrary distance $\rho$ from the center has components of velocity both parallel and perpendicular to the axis of the rod, which generate differential drag forces in the opposite direction. The plane of rotation is tilted away from the horizontal by an angle $\theta$, resulting in an enhanced drag coefficient for the segments of the rod nearest to the lower surface. The height of the center of the rod above the floor is denoted by $h_0$. The perspective image (c) shows the rod as it travels through a fraction of its rotation in the vicinity of the floor. Credit: Jerome Carpenter.

Using experimentally determined values or value ranges for $L$, $r$, $\theta$, and $h_0$ we evaluate the translational velocity vs. angular velocity data with respect to our model. $L$ and $r$ are experimentally determined to be $5.5 \pm 0.1 \, \mu m$ and $150 \pm 30 \, nm$, respectively.
respectively (SEM imaging). An average rod height of $h_0 = 1.0 \pm 0.2 \, \mu m$ is determined using a piezo stage (Mad City Labs, Madison, Wisconsin) to focus on rods in motion, then compare these heights with nearby rods adhered to the sample floor. We determine a range for $\theta$ by analysis of rod apparent length during each frame of a rod rotation, which yields an upper bound of $\theta_{max} = 20^\circ$.

Using the specified $L$, $r$, and $h_0$ values we evaluate $v_t$ vs. $\omega$ for $\theta = 5^\circ$ and $15^\circ$. For $\theta = 5^\circ$ and $15^\circ$, $v_t = 0.05\omega$ and $0.26\omega$, respectively (solid lines shown in Figure 4.8). Our rods move with $v_t = 0.11\omega$, which is in agreement with the theoretical results shown in Figure 4.8. This crawling mechanism allows for continuous control over nanorod position, as fractions of a rotation can be used to achieve high resolution, incremental advances in position. If the notion of individual steps are applied to these Janus nanorod crawlers then a rotation of $\pi$ radians would serve as a useful representation of a single step. Using this measure of a step we can attribute a step size of $350 \, \text{nm/step}$ to the crawling mechanism, as implemented by our Janus crawlers. Changing the rod length, radius, and, most significantly, angle with respect to the floor, allows for the step length to be tuned to meet a specific set of micro-manipulation requirements.

4.3.4 Using Janus nanorods for payload capture and manipulation

One intriguing potential application of nanorod translation is the ability to manipulate payloads in solution. To that end, we use Janus rods labeled on one side with 1,6-hexanediethiol to capture and translate a 1 $\mu m$ metal-coated polystyrene (PS)
Figure 4.8: Measured translational (crawling) velocities of 5 magnetic Janus nanorods as a function of their angular velocities (data points), demonstrating a linear relationship. The dashed line is a linear fit to the data, with \( \nu_t = 0.11 \omega \). The solid lines represent the average translational velocity as a function of angular velocity, as predicted by our model, for rods with \( \theta = 15^\circ \) and \( 5^\circ \). These relationships are calculated for rods sitting \( h_0 = 0.9 \ \mu m \) above the glass floor, as determined by the average of 9 rod heights.

Briefly, we prepare Janus rods for bead capture by gentle vortexing of rods in 1,6-hexanethiol (1mM in ethanol), followed by centrifugation and subsequent rinsing (10×) in ethanol. PS beads (1 \( \mu m \) diameter, Molecular Probes) are prepared by first diluting stock solutions 1/1000 in ethanol (~\( 4.2 \times 10^{-3} \) % solids). These dilutions are sonicated for 30 minutes, deposited onto plasma-cleaned glass slides and sputter-coated with ~10 nm 80Au-20Pd films. Beads are collected via substrate sonication in ethanol (20 s, 12 W), after which they are centrifuged and rinsed. We load a sample
chamber with both Janus nanorods and PS beads and controllably steer a chosen rod towards a PS bead suspended in solution near the surface (Figure 4.10). Coarse rod manipulations are performed at translational velocities of $\sim 2 - 4 \ \mu m/s \ (\sim 20 - 40 \ \text{rad/s})$. Once near the PS bead the rod angular velocity is slowed ($\sim 5 - 10 \ \text{rad/s}$) to allow for fine positioning of the rod. When contact is made between the thiol coated face of the rod and the bead (Figure 4.10c), a covalent bond is formed between the AuPd layer of the PS bead and the exposed thiol groups on the Au segment of the Janus rod. Manipulation of chemically unmodified Janus rods into contact with AuPd-coated PS beads results in no observable interaction. After capture of a PS bead we observe no change in nanorod response to the rotating magnetic field, indicating that the presence of a bead payload does not perturb the translation mechanism measurably.

Figure 4.9: Schematic of Janus nanorod surface functionalization for bead capture.
Figure 4.10: Dithiol-labeled Au-Ni Janus rod (lower arrow, (a)) approaches a pair of AuPd coated polystyrene beads (upper arrow, (a))(a-b), captures the beads via covalent linkage attached to the Au side of the rod (c), and is then manipulated towards the upper right corner of the frame (d), then towards the lower left corner of the frame (e-f). The broken red line indicates the path of the Janus nanorod. Scale bar represents 20 µm.

Video: Thiol-gold chemistry allows for capture of a Au-Pd-coated microbead.
4.3.5 Experimental work using magnetic rods to manipulate cells

As a final, biologically relevant example of controllable manipulation, we also demonstrate single cell translation using our magnetic Janus nanorods. Although this cell guidance mechanism does not rely on Janus nanorod crawling, like nanorod crawling it allows for multidirectional translation of individual cells with spatial resolution (hundreds of nanometers) significantly smaller than the dimensions of the cell being manipulated (10-15 µm). In general, a sphere rotating near but not in direct contact with a surface will translate in a plane parallel to that surface (Goldman et al., 1967; Blake, 1971; Reichert and Stark, 2004; Kim et al., 2010). We use HBE-16 cells (see Cell Methods at the end of this chapter) with diameters between 10 and 15 µm and couple our nanorods to them via nonspecific binding to membrane proteins. After cell-nanorod mixing in PBS the solution is deposited into PDMS wells for magnetic manipulation. During experimentation, cell-nanorod constructs settle near the surface with a variety of orientations. Optical microscopy indicates that HBE-16 cells in solution are indeed approximately spherical. We suggest that this sphere-spinning mechanism, as motivated by the field-aligning behavior of the Janus nanorods, leads to controllable single-cell translational manipulation near the surface of the glass slide substrate. Indeed, in all cases rotation of the actuating permanent magnet results in cell rotation and subsequent translation. While detailed modeling of this translational mechanism is beyond the scope of this work, it is important to note that the resulting directed cell translation, like nanorod crawling, exhibits high resolution (less than the nanorod length) and controllability (Figure 4.11). As the
efficacy of this method relies heavily on the magnetization $M$ of the nanorod, rods with larger amounts of magnetic material are preferred for the manipulation of cell-sized payloads. For example, pure Ni rods would prove more effective at single cell manipulation as the field-aligning behavior of our Janus rods utilizes only a 50 nm thick hemispherical layer of magnetic material.

One advantage of Janus rods is their highly tunable and user-specific composition. While Ni is not well tolerated by cells, our technique for the fabrication of high aspect-ratio magnetic Janus rods makes it possible to conceal the ferromagnetic Ni layer by integrating materials with decreased cytotoxicity. Ultimately, materials more suitable for long-term biocompatibility, such as hydroxyapatite (Balasundaram et al., 2021).
Figure 4.11: A human bronchial epithelial cell being manipulated by a single nanorod. In (a - c), magnet rotation is clockwise and the cell moves towards the top right of the field of view. In (d - f), the magnet position is changed and direction is reversed and, consequently the cell is manipulated towards the lower right of the field of view. Three frames (g) show the changing orientation of the Janus rod (arrows) relative to the cell under the rotating magnetic field. Scale bars represent 30 µm (a-f) and 5 µm (g).

et al., 2006), may serve as candidates for Janus rod fabrication. We have demonstrated maneuverability of magnetically actuable magnetic nanorod swimmers and demonstrated their ability to manipulate payloads with sizes of 1 µm and ∼10 µm.
Within microfluidic settings this active translational mechanism is a simple method for transporting inorganic and biological payloads alike. Ozin et al. proposed that synthetic nanomachines may be useful as nanoscale agitators for the stirring of liquids in microfluidic channels or near the surfaces of electrodes for local enhancement of the diffusion process (Ozin et al., 2005). Due to their active mode of transport (rotational manipulation) our Janus nanorod swimmers have the potential to operate as dual-functioning transporters and micromixers, a capability not possible with simple spheres translating by magnetic force application. For some systems in which the rate of a process is typically prescribed by normal diffusion, the rotational component of this transport mechanism may also result in rate changes (increases) as a result of the induced agitation of the surrounding environment (assuming the diffusion process is sufficiently slow).

While previous reports on manipulating individual magnetic wires (Fan et al., 2004; Fan et al., 2005; Fan et al., 2006; Fan et al., 2008) and assemblies of wires (Bentley et al., 2004; Bentley et al., 2005; Edwards et al., 2006; Keshoju et al., 2007; Sun et al., 2008) have contributed significantly to the understanding of magnetically active rods and wires, the rotation-induced translational motion of rods near surfaces offers an additional application for these structures. Other rotating low-Reynolds number swimmers have been produced by joining micron-sized paramagnetic beads and rotating these structures in the vicinity of a wall; these reports rely on polymeric beads with minimum feature dimensions greater than or equal to 1 µm (Tierno et al., 2008a; Tierno et al., 2010; Tierno et al., 2008b; Tierno et al., 2009). Magnetic nanorod
swimmers are easy to fabricate and can incorporate insulating, semiconducting, metallic, and polymeric materials along the rod axis (Hurst et al., 2006) or preferentially along each side of the rod, as demonstrated here. A magnetically responsive rod segment is all that is required, and significant multifunctionality can be realized, for example, by implementing nanorod crawling using complex rod structures such as the gold-polypyrrole-nickel-gold rods fabricated by Bangar et al. (Bangar et al., 2009). Additionally, nanorod swimmers can be created with highly tunable aspect ratios, whereas microbead swimmers are confined to incremental changes in swimmer length, the increment being defined by an individual bead diameter.

Previous researchers have used optical traps (Pauzauskie et al., 2006), electric fields (Fan et al., 2008), and magnetic fields and field gradients (Hangarter et al., 2007; Hangarter and Myung, 2005) to manipulate nanowires. In the case of magnetic field translational manipulation, effective translation requires that a field gradient be present, as a field alone only results in aligning of the wires with the field direction. Importantly, our method allows for translation manipulation in the absence of magnetic field gradients, as the only relevant magnetic interaction is the field-aligning behavior of the rod.

Although the nanorod-cell attachments we utilize are nonspecific, future biomedical applications of magnetic Janus nanorods may make use of the available surface chemistries to differentially label each side of the Janus rod with various molecules, proteins, or even other nanoparticles. For example, binding polyethylene glycol (PEG, a commonly used protein adhesion inhibitor) and fibronectin (an extracellu-
lar matrix glycoprotein which binds cell membrane integrins) to the two sides of a Janus nanorod may result in side-specific cell-nanorod binding. Future applications of these Janus rods may include more elaborate face-specific surface functionalization, magnetic protein and cell separation, and the manipulation of other types of payloads such as viruses and bacteria. Also, these Janus rods make it possible to manipulate complexly structured, multisegmented insulating and semiconductor nanorods and nanowires, adding functionality to the ever-expanding high aspect ratio nanomaterials toolbox.

**Cell Methods:** Human transformed bronchial epithelial cells (HBE-16) are grown in T-25 cell culture flasks. The cells are washed with PBS twice per week and maintained with DMEM/F12 with 5% fetal bovine serum. HBE-16 cells are routinely passaged upon reaching confluence. In preparation for Janus nanorod manipulation, these cells are trypsinized, spun into a pellet and resuspended in DMEM/F12 at a concentration of about 2 million cells/ml. Cells are incubated with Janus nanorods under gentle vortex mixing for 1 hour prior to magnetic manipulation. The manipulation experiments presented here make use of nonspecific cell-nanorod binding (Hultgren et al., 2003), which mechanically couples the cell membrane to the rods. Although not all cell-nanorod interactions result in irreversible cell binding and particle adhesion, successfully bound nanorods remain intact for the duration of these cell manipulation experiments.
4.4 Conclusion

In this chapter I have discussed my implementation of template-guided electrodeposition for the fabrication of various materials, including multisegmented rods. I have demonstrated the fabrication of novel magnetic Janus nanorods, and have rotationally actuated these rods near a surface to produce translational motion via hydrodynamic swimming. Additional functionalization of these rods enabled covalent cargo capture via gold-thiol chemistry. Rotating Janus rods attached to the surfaces of cells was shown to induce cell rotation which, when performed near the sample chamber floor, also resulted in directed translation. This method for making magnetic nanomaterials will be later utilized in Chapter 6.
Chapter 5

Macromolecule Diffusion in Extracellular Matrix

5.1 Introduction

The previous two chapters have dealt with inorganic particles, their fabrication, and their application to various systems (transfection to cells for oligonucleotide delivery in vitro, bead capture, and single cell manipulation, for example). In these experiments the principal materials involved were the inorganic particles, buffer, and cells. Most particle transport detailed up to now has been through buffer. In this chapter I initiate a second segment of this thesis in which I study particle transport through a hydrogel, including passive (diffusive) transport (this chapter) as well as driven (magnetofective) transport (Chapter 6).

In this chapter I discuss experiments for determining how variously-sized molecules diffuse through Matrigel. The results obtained are unique in that they are the first
experiments which interpret a single set of Matrigel diffusion data using the Renkin, Ogston, and Phillips models for diffusion in porous media. These models have been applied to various synthetic and biological materials, however no report has assessed Matrigel using the confluence of these methods. By doing so it is possible to decipher various aspects of the models, and how they capture or fail at capturing the important parameters for studying diffusion in Matrigel. As such, this represents a new, more complete method for analyzing Matrigel. Additionally, this technique of multi-model analysis offers an example to others wishing to perform similar studies using other materials.

Matrigel is a significant material to understand, as it is widely accepted as a cell culture material and commonly used as a material for performing cell invasion assays to assess the metastatic potential of cells, both those excised from patients as well as those modified within laboratory settings so as to study the impact of drugs, proteins, and cell characteristics (such as membrane mechanics) on the likelihood of cell metastasis. Significant events take place in the extracellular environment: cell-cell signaling, cell motility, as well as small molecule / therapeutic diffusion. As such, understanding diffusion in this material is crucial. And because Matrigel is so ubiquitous in laboratories around the world, an understanding of its size-specific diffusion properties warrants study.
5.2 Motivation

The extracellular environment plays a significant role in cellular processes (Jain and Stylianopoulos, 2010). It has been heavily implicated in cell signaling (Guillaume-Gentil et al., 2010) and cell differentiation (Discher et al., 2005), as well as the hindrance of macromolecule / nanoparticle diffusive transport (Berk et al., 1993a; Berk et al., 1993b; Au et al., 2001; Au et al., 2002; Jang et al., 2003; Burke and Pun, 2008; Goodman et al., 2008). Specifically, the extracellular matrix (ECM) is a rich and complex system of biopolymers capable of size and charge exclusion on the scale of tens to thousands of nanometers. Within a tumor, successful nanoparticle and macromolecule treatment efficacy is dependent upon wide-spread distribution of a delivery vector throughout the tumor volume (Jain and Stylianopoulos, 2010). Wide-spread distribution requires transport through cells as well as the ECM. The ECM frequently limits extensive distribution of macromolecule and nanoparticle chemotherapeutics (Au et al., 2001; Goodman et al., 2008; Eikenes et al., 2010) due to its dense and fibrous nature and, thus, an understanding of the structure, function, and probe diffusive properties in the material is important for addressing such problems. This chapter will briefly introduce the structure and function of the ECM, discuss probe diffusion properties in this material, and detail diffusion experiments performed on an ECM material in vitro. Importantly, this chapter will explore modeling the ECM employing the fiber-matrix framework for diffusion in porous media using probes with radii ranging from 3.5 to 24.6 nm. This size range is significant, as
it covers at one end the smallest magnetic particles currently available for potential use in magnetic drug targeting systems (more on that in Chapter 6), and moves into the size regime which has been considered relevant for nanoparticles and proteins (tens of nanometers). Specifically, this chapter will use the measurement of $D_{gel}/D_0$ to determine an estimate for the pore size of a Matrigel sample. This is the first time $D_{gel}/D_0$ for an entire series of dextran sizes has been used to determine pore size for Matrigel. This series allows comparison of how differently sized probes experience differently sized pores, on average.

This entire chapter exists to inform questions regarding the pore size of the ECM. How much diffusion suppression does Matrigel provide? More specifically, how much diffusion suppression does Matrigel provide for a given probe size? On average, how large are the pores in samples of Matrigel? And how do these values compare with reported values for excised and in vivo ECM? Can Matrigel reasonably be used to approximate ECM diffusion? Of course, the field of transport through fibrous / porous media is well established and several model frameworks exist for understanding structure-property relationships within such media. While Katz et al. have studied the permeability of Matrigel (Katz et al., 1992; Katz and Lamarche, 1994), no reports currently exist on the size dependent diffusion in Matrigel. In the following paragraphs I will introduce the relevant models and discuss their application to diffusion in Matrigel.
5.3 Structure and function of the extracellular matrix

A pervasive theme in the field of materials science is the nature of a material’s structure, and how that structure relates to the properties of that material. Although this thinking was originally applied to inorganic materials having ordered lattice arrays of atoms involving interstitial atoms, vacancies, and impurities, this structure-properties framework of evaluating and understanding materials is no less relevant in the realm of organic and biological materials. Specifically, in biopolymer systems which contain both solid and liquid phase materials (fibrous protein bundles, uncrosslinked, freely diffusing protein, as well as buffer), an understanding of the structure-property relationship leads to a better understanding of the material’s in vivo performance (Peppas et al., 2000; Slaughter et al., 2009). Understanding the function of the ECM requires a basic physical structural model of the structure of the matrix.

The interstitial matrix is found between the various cells in the intercellular spaces within in tumor; the ECM includes the interstitial matrix and the basement membrane supporting the epithelium or epithelium cell layer. The interstitial matrix is generally composed of gels of polysaccharides and fibrous proteins and performs the same general function as the ECM — namely, to provide support and signaling pathways for the cells residing within these matrices. Generally speaking, within a localized tumor the ECM volume is the tumor volume, minus the tumor cell volume and vascular space volume. In rats, the percentage of a tumor attributed
Figure 5.1: Assembly of the ECM / basement membrane (LeBleu et al., 2007). Importantly, this image depicts the role of laminin-laminin bonds, collagen-collagen linkages, as well as laminin-collagen binding sites.
to the interstitial matrix ranges from 13% for skeletal muscle tumors to 60% for fibrosarcoma; in humans, gliomas frequently present with interstitial components from 20–40% and meningiomas from 13–15% (Jain, 1987). These results indicate that the ECM has the potential to play a significant role in pharmaceutical distribution within these tumors.

5.3.1 ECM structure

The ECM is a highly evolved material and, as such, there is an entire array of conditions which manifest from abnormalities in its structure. Diabetes mellitus (Yurchenco and O’Rear, 1993), Alport syndrome (Barker et al., 1990), and epidermolysis bullosa (Bruckner-Tuderman et al., 1989) are all conditions in which detrimental health outcomes are due to unusually large pores in the basement membrane. Because the current chapter and the following chapter both deal with transport through this material, a basic understanding of the matrix structure is needed.

The ECM is essentially a two-phase material composed of a gel in which fibrous proteins are suspended. Although in vivo composition varies depending on location, cell type, and tumor developmental stage, the matrix generally consists of a variety of polysaccharides and proteins which are secreted and assembled by the cells which the ECM surrounds. The ECM consists of two main types of macromolecules: (1) long fibrous proteins, such as collagen, laminin, elastin, and fibronectin, and (2) polysaccharide chains called glycosaminoglycans (GAGs, which are typically covalently bound to proteins, making them proteoglycans). These long fibrous protein
molecules typically perform structural and adhesive functions within the matrix. The principal components of the fibrous and elastic phase of the ECM are collagen, laminin, and elastin. The building block of these structural units is the collagen molecule, a 1.5 nm wide, 300 nm long cylindrically shaped molecule consisting of 3 alpha peptide chains twisted around one another in a triple helix formation. These fibrous proteins provide tensile strength for the matrix and organize in space the ECM and its contained cells. There are 14 different types of collagen molecules, however a typical ECM is largely composed of a small number of types of collagens. Type IV collagen is particularly prevalent in mammalian ECMs.

**Collagen IV:** Collagen IV is the most prevalent protein in the ECM and is composed of three polypeptide chains (two $\alpha 1$(IV) chains and one $\alpha 2$(IV) chain) and terminates in a globular domain called the NC1 domain. In the Engelbreth-Holm-Swarm (EHS) tumor material the collagen is non-reversibly crosslinked via disulfide bonds and lysyl oxidase-derived bonds formed primarily at the amino and carboxyl terminals of the fiber. Using only these bonds, the apparent structure of the collagen IV network would be that of a square lattice of collagen with lattice dimensions of 800 nm created by flexible triple-helix filaments joined at very regular intervals. However, Yurchenco and Furthmayr first observed the existence of lateral collagen-collagen associations in human amnion matrix material via TEM (Yurchenco and Furthmayr, 1984). These lateral associations are concentration- and temperature-dependent, exhibit thermally reversible properties (Yurchenco and O’Rear, 1993), and were shown to exist in the EHS tumor matrix as well (Yurchenco and Ruben,
Importantly, fiber assemblies were directly observed via electron microscopy, and the average fiber diameter was similar in both human amnion and mouse EHS tumor matrix materials. Similar fiber diameters (2.5–7 nm), spacing between vertices (41–44 nm on average), as well as branch point complexity (3–5 arm branch points) were observed for both matrices (Yurchenco and O’Rear, 1993).

**Laminin:** Laminin, the second most common ECM protein, is a glycoprotein made up of three polypeptide chains (A, B1, B2). These chains take on a four-armed arrangement, with three short arms (two ~35 nm B-chain arms, one ~50 nm A-chain arm) and one long arm (~75 nm) (Beck et al., 1990). Each arm contains anywhere from one to three globular domains, each of which is utilized in self-assembly, during which individual laminin proteins bond at their globular domains to form a mesh-like network. Laminin self-assembly requires a minimum 0.1 µM concentration and is completely thermally reversible, meaning that at 4°C the polymer dissociates back into its constituent monomer components. Importantly, laminin assembles in a variety of end-to-end contacts, including short-to-short, long-to-long, and short-to-long arm linkages (Yurchenco et al., 1985); the organizational structure of the laminin network resembles a polygonal lattice of flexible fibers with an average fiber length of 30 nm (Yurchenco and Schittny, 1990).

**Glycosaminoglycans and Proteoglycans:** The second ECM phase, composed of glycosaminoglycans and proteoglycans, forms a soft, viscous gel through which small molecules (such as nutrients and hormones) can readily be transported. It is composed primarily of hyluronate and proteoglycans, with molecular sizes in the
Figure 5.2: TEM images of collagen and laminin, taken with permission from the landmark work of Yurchenco and Schitity (Yurchenco and Schitny, 1990).
range of 0.5 – 5 nm. This gel also resists compressive forces which would otherwise permanently deform the matrix and the residing cells. A highly hydrophilic material, it has a high negative charge density and readily swells, imparting a viscoelastic element to the matrix. The principal components of this gel are heparan, heparan sulfate, and chondroitin sulfate. Heparan has been shown to bind to collagen at both globular domains as well as along the collagen chain; additionally, at very high concentrations (hundreds of $\mu$g/ml) heparan actively prevents lateral associations between collagen chains, thus reducing the network density (Yurchenco and O’Rear, 1993).

**Other Components:** Aside from the fiber-forming collagen IV and laminin and the viscous gel of glycosaminoglycans and proteoglycans, there also exists a distinct set of proteins which serve as linking proteins for the various ECM components. Entactin / nidogen is the primary protein operating in this function. Entactin is a 20 nm long protein (Carlin et al., 1981; Timpl et al., 1983) which typically exists in molar quantities equivalent to laminin and is extracted intact with laminin (Paulsson et al., 1987), despite it not being required for laminin polymerization. Fox et al. proved that indeed nidogen ties the laminin and collagen networks together at specific points along the collagen IV fiber (Fox et al., 1991).

**Matrix Organization:** As has been discussed, the matrix can be morphologically considered an elaborate network of filaments and linking struts. Using only the filamentous components of the matrix (collagen and laminin) one cannot explain the filtration properties of the extracellular matrix or glomerular basement membrane.
However, considering the additional network linkages formed among filamentous proteins combined with heparan sulfate and linking nidogen it becomes more clear how filtration of small proteins is accomplished.

### 5.3.2 ECM function

The ECM performs a host of functions, and most of these functions are explicitly possible due to its structure. The three most fundamental functions of the ECM are (1) providing cell surface anchorage, (3) cell organization, and (3) regulating intercellular communications. Because of its role in cell adhesion and organization, remodeling of the ECM is a perpetual process which becomes more intense during wound healing.

As has been shown, mechanical properties of the cellular microenvironment are extremely important for cell function and, in the case of stem cells, differentiation (Discher et al., 2005). Specifically, in the case of tumor ECM, the matrix provides a substantial barrier to long range directed or diffusive transport (Swabb et al., 1974; Netti et al., 2000; Jain and Stylianopoulos, 2010).

### 5.3.3 Matrigel

Matrigel was invented during the early 1980s by researchers at the National Institutes of Health (Kleinman et al., 1982). Since that time it has become the standard material for supporting cell morphogenesis, differentiation, and tumor growth. In 1986 Kramer et al. showed that HT1080 tumor cells aggressively invaded Matrigel sam-
ples, while healthy fibroblasts simply formed small non-invasive colonies (Kramer et al., 1986). By 1987 Albini et al. published their landmark finding that Matrigel could be used as an invasion assay material in experiments which differentiate cancer cells from healthy cells based on their proclivity to invade a Matrigel sample (Albini et al., 1987). Matrigel has also been highly instrumental in stem cell differentiation (Xu et al., 2001; Kohen et al., 2009; Hughes et al., 2010).

Matrigel is a protein-rich, commercially available material sold through BD Biosciences. It consists of constituent molecules extracted from the Engelbreth-Holm-Swarm murine sarcoma (EHS sarcoma or EHS tumor) and forms a soluble, sterile, three-dimensional gel at 37°C. The concentration of Matrigel varies slightly among individual lots and, consequently, we use a concentration normalized form (8.75 mg/ml). This matrix is composed of approximately 56% laminin, 31% collagen IV, and 8% entactin (reference: http://www.bdbiosciences.com/cellculture/ecm/), although some reports differ (McCarty and Johnson, 2007; Fissell et al., 2009). Approximately 80% of the protein in Matrigel forms a gel.

Recently, the mechanical (Soofi et al., 2009; Reed et al., 2009) and permeability (Fissell et al., 2009) properties of Matrigel have been studied. Soofi et al. reported a modulus of 450 Pa for Matrigel at physiological conditions. Reed et al. found that Matrigel plastically deforms at stress levels similar to those exerted by cells during tractional force application, while Fissell et al. reported on size and pressure dependent sieving through Matrigel. Despite its longstanding commercial availability, many of the physical and materials properties of Matrigel have only recently been
studied in depth. This chapter contributes to an understanding of size-dependent
diffusion in Matrigel by applying three different models for diffusion in porous me-
dia to the gel and comparing how model predictions fare with respect to the data
and with respect to one another. Specifically, this chapter compares size-dependent
diffusion in Matrigel with literature values for diffusion in other tumor matrices.
Also, here I will establish values for $a_{diff}$ and $b_{diff}$ in the power law equation relating
dextran molecular weight $M_w$ with diffusion coefficient, $D = a_{diff}(M_w)^{b_{diff}}$, first put
forth by Nugent and Jain (Nugent and Jain, 1984). This equation can be fit to myriad
cases of size-dependent diffusion in biological material and the data in this chapter
represents a first instance of Matrigel diffusion data being used to establish unique
and original values for $a_{diff,Matrigel}$ and $b_{diff,Matrigel}$.

5.4 A primer on diffusion in biological materials

Diffusion is a ubiquitous process in biological systems; its importance in biolog-
ical function cannot be overstated. As discussed previously, once macromolecules /
nanoparticles leave the vasculature of the tumor their only mode of transport is diffu-
sive. Consequently, a variety of systems have been used to study diffusion and relate
specific parameters observed in vivo with components in model systems. These
systems include entirely manufactured environments (such as solutions of synthetic
polymers), in vivo systems (such as the experiments performed by Nicholson et al.
involving diffusion in live mouse cranial windows (Thorne et al., 2008)), and every-
thing in between. In this section I’ll review the principal components necessary for understanding diffusion in fibrous biological materials and introduce several of the major models used in understanding the impact of various component values on diffusion in biological materials, denoted here as $D_{gel}$ (whereas $D_0$ is used to refer to diffusion in buffer).

Now it is important to quickly summarize the possible states in which a probe is allowed to exist in a biopolymer material. First there is the size effect. Particles can be much smaller than, on the order of, or much larger than the average pore size of the matrix. Generally speaking, particles much smaller than the average pore size will diffuse relatively freely in the material. For biopolymers having buffer-like solution between the fibers, this will result in $D_{gel}/D_0 \approx 1$ (no diffusion suppression). Particles much larger than the average pore size will be sterically caged by the fibers of the material, with $D_{gel}/D_0 \approx 0$ (complete diffusion suppression). Particles on the order of the mesh size will experience some diffusion suppression, but will generally still be able to traverse large distances (several probe diameters) over a reasonably observable time period (tens to hundreds of seconds) given a buffer-like interstitial fluid. Commonly, this results in $0.01 < D_{gel} / D_0 < 0.99$. The level of suppression allows us to estimate average pore dimensions. In addition to size effects there are surface charge effects which may need to be considered for probes which exhibit substantial surface charge. A variety of surface charges exist in most biopolymer systems. Recent work has elucidated the relationship between probe charge and diffusion suppression in biopolymer systems (Valentine et al., 2004; Lai et al., 2007b;
Suh et al., 2007; Lieleg et al., 2009; Rusu et al., 2010). Additionally, proteins offer up a wide range of functional groups capable of interacting with probes. As a result, non-binding, transient binding-unbinding, and permanent binding are all possible for non-neutral probes, particularly larger probes.

As magnetophoretic applications are the main goals of this dissertation, the study of surface charge and its effect on particle binding/unbinding is beyond the scope of this document. However, Valentine first suggested that grafting PEG onto the surfaces of particles serves to inhibit non-specific protein binding (Valentine et al., 2004), and since then Lai et al. have shown this to hold true in mucus (Lai et al., 2007b; Lai et al., 2007a) and Lieleg et al. have shown similar charge-selectivity in ECM (Lieleg et al., 2009). Because non-adherent dextran molecules are used for the experiments in this chapter, we assume no binding/un-binding reactions take place and consider the motion of dextrans in Matrigel as completely diffusive. Immobile fractions observed during FRAP experiments were typically less than 10% and can reasonably be attributed to the steric caging effect of the ECM gel on the probes. As has been discussed, the ECM exists as a complex spatial arrangement of fibers and linking proteins. A future section will discuss how this complexity may contribute to varying matrix pore and fiber dimensions.

Standard diffusion in a purely Newtonian fluid (often called Brownian diffusion) is described by the Stokes-Einstein relationship (Einstein, 1905) ¹

¹Historical note: Interestingly, a similar result was obtained by William Sutherland in 1904, and published in 1905 (Sutherland, 1905). The two manuscripts were submitted within months of one another, Sutherland’s in March of 1905, Einstein’s that May (Pais, 1982; Hänggi and Marchesoni, 2005).
\[ D = \frac{k_B T}{f} = \frac{k_B T}{3\pi \eta d} \] (5.1)

where \( k_B \) is the Boltzman constant, and \( T \) is the absolute temperature. \( D \) represents the diffusion coefficient of a spherical probe with diameter \( d \), and \( \eta \) is the viscosity of the fluid. It should be noted that, for non-spherical probes, \( f \) should be replaced by the Stokes drag formula specific to the given probe geometry (Happel and Brenner, 1983). Einstein’s work also details how \( D \) satisfies the bulk diffusion quantity of Fick’s Laws for homogenous, time-invariant materials.

Biological systems, of course, do not meet the conditions of homogeneity, nor time-constancy. Polymer systems are inherently heterogenous, with some space being solvent rich, and other space being solute rich / polymer rich. Specifically, in biological polymers such as mucus, the cytoskeleton, and the extracellular matrix there are biopolymeric components which can be considered as fibers having some average fiber dimensions, as well as a solvent in which these fibers exist, frequently having a viscosity on the order of water or buffer. Because biopolymer systems do not meet the conditions of homogeneity and time-invariance necessary for using Equation 5.1, other models must be established. Specifically, the Stokes-Einstein relationship assumes that the probe in question is allowed to diffuse through the entirety of the sample. As discussed, the fibrous nature of many biopolymer systems require a treatment which takes into account this unavailable space (the space occupied by fibers).
**Renkin model:** In 1954 Renkin published a seminal paper describing a method for using only values for diffusion suppression ($D_{gel} / D_0$) and the probe dimension to obtain an approximate pore size in porous media (Renkin, 1954). Renkin’s work offered additional experimental validation to a theory of restricted diffusion and molecular sieving through membrane-like materials such as living capillaries presented by Pappenheimer only a few years prior (Pappenheimer et al., 1951; Pappenheimer, 1953). Specifically, Renkin’s experiments involved the sieving of small solutes (2 – 6 Å) through cellulose membranes. Data was analyzed and interpreted by simplifying the membrane to be an assembly of close-packed cylindrical pores. The theory has been invoked when explaining antibody diffusion in human cervical mucus (Saltzman et al., 1994), diffusion in gels (Pluen et al., 1999), protein transport through membranes (Blocker et al., 1999), dye-sensitized solar cells (Hardin et al., 2010), transport pathways through rat skin (Seki et al., 2010), as well as several other scenarios.

Renkin’s relation describes the restricted diffusion of spherical molecules in cylindrical pores as

$$
\frac{D_{gel}}{D_0} = (1 - \lambda_R)^2(1 - 2.1044\lambda_R + 2.089\lambda_R^3 - 0.948\lambda_R^5),
$$

(5.2)

where $\lambda_R = R_H / r_p$, $R_H$ being the diffusing probe hydrodynamic radius, and $r_p$ being the pore radius (Renkin, 1954). In section 5.7 I will apply the Renkin equation to dextran diffusion in Matrigel and obtain values for $r_p$ from experimentally determined...
diffusion coefficients. Specifically, the predictions for Renkin pore dimension based on the experimental results obtained using differently sized probes will be discussed.

Although the Renkin model relies on an empirical fit to an idealized scenario, recent work suggests its broad applicability in biopolymer systems including collagen, cervical mucus, and gelatin gels (Saltzman et al., 1994; Cu and Saltzman, 2009). Here I apply it as a means of going from diffusion suppression data directly to pore size without any knowledge of fiber diameter or volume fraction. In section 5.7 I will apply each different size of dextran probe diffusion data to the Renkin model in order to obtain a range of predicted pore dimensions, and discuss the reasoning behind the predicted pore dimensions from the macromolecule’s eye view.

**Ogston model:** While the Renkin model considers probes traveling through cylindrical pores, Ogston proposed a solution to solute transport through a network of uniformly thick, randomly oriented, cylindrical fibers (Ogston et al., 1973). Ogston’s model assumes that there is no hydrodynamic drag between the cylindrical matrix fibers and the diffusing solute. The dimension and frequency (concentration) of the fibers are what dictate diffusion suppression, as the presence of fibers prevents the probe from moving. This is an entirely steric effect model in which diffusion suppression is caused entirely by the matrix fibers physically inhibiting the motion of a diffusing particle as the particle comes into direct contact with the fibers. In Ogston’s treatment the fibers are considered to have lengths significantly longer than the probe dimension $d_p$, and diffusion suppression for a given probe can be calculated based on the fiber diameter $d_t$, probe diameter $d_p$, and volume fraction $\phi$ of the gel (the
fractional volume which is occupied by fibers, with $0 < \phi < 1$). The relationship is captured in the equation

$$\frac{D_{gel}}{D_0} = \exp\left(-\phi^{1/2} \frac{r_s}{r_f}\right),$$

where $r_s$ is the solute radius and $r_f$ is the fiber radius.

Ogston’s model for diffusion in porous media has been applied to transport in basement membrane (Edwards et al., 1997), mucus (Cu and Saltzman, 2009), fibrin (Spero, 2010), and several other polymers and biomaterials. While in each of these more recent cases discrepancies were observed between the experimental data and the Ogston model, Ogston’s work serves as an effective tool for baseline estimation of matrix parameters. This model could potentially be useful here if parameters such as $d_i$ and $\phi$ are known. Determining $\phi$ for such a complex confluence of biopolymers is not straightforward. Later in section 5.7 I will use the collected data ($D_{gel}$) and the known solute radii ($r_s$), and a range of fiber radius ($r_f$) values to (coarsely) estimate the volume fraction $\phi$.

**Phillips model:** Neither the Renkin nor the Ogston models deal with hydrodynamic effects between the solute and the matrix fibers. A model including both steric and hydrodynamic effects was synthesized by Phillips (Phillips, 2000), drawing heavily from previous work in the field (Brady, 1994; Johnson et al., 1996; Clague and Phillips, 1996), and is captured by a two-term equation:
\[
\frac{D_{\text{gel}}}{D_0} = FS(f),
\]  

(5.4)

where

\[
F = \exp(-a\phi^b),
\]  

(5.5)

\[
S(f) = \exp(-0.84f^{1.09}),
\]  

(5.6)

\[
a = 3.727 - 2.460\lambda_P + 0.822\lambda_P^2,
\]  

(5.7)

\[
b = 0.358 + 0.366\lambda_P - 0.0939\lambda_P^2,
\]  

(5.8)

\[
f = (1 + \frac{r_s}{r_f})^2\phi,
\]  

(5.9)

\[
\lambda_P = \frac{r_f}{r_s}.
\]  

(5.10)

In the Phillips model, \(F\) represents the hydrodynamic effects and \(S(f)\) represents steric effects. The separation of these two effects significantly aids in understanding how each effect contributes to diffusion suppression for a given set of fiber/matrix/probe parameters. An additional benefit of the Phillips model is that the hydrodynamic factor, \(F\), has its roots in the Brinkman effective medium theory (Brinkman, 1947), and thus is related to the hydraulic permeability, \(k\), via

\[
F = (1 + \frac{r_s}{\sqrt{k}} + \frac{1}{9}(\frac{r_s}{\sqrt{k}})^2)^{-1}.
\]  

(5.11)

As such, estimations of \(F\) and \(S(f)\), along with information regarding \(D_{\text{gel}}/D_0\) can...
yield approximations for hydraulic permeability without experimental permeability data.

**Tortuosity in ECM materials:** Additionally, neuroscience researchers interested in the diffusion properties and structure of brain extracellular space make use of a concept called tortuosity, $\lambda_{tortuous}$, via the equation $\lambda_{tortuous} = \sqrt{D_0/D_{gel}}$. This relationship, first proposed by Harris and Burn (Harris and Burn, 1949), is intrinsically a coarse measurement which encompasses all causes for diffusion suppression (steric, hydrodynamic, caging (extreme steric), transient cell or matrix binding, and non-specific charge interactions (Thorne and Nicholson, 2006; Sykova and Nicholson, 2008)). As such, this measure is primarily used as a comparative gauge for analyzing diffusion suppression differences among matrix samples.

In section 5.7 each of these methods will be discussed with respect to the collected fluorescence recovery after photobleaching data.

**Macromolecule transport in extracellular matrix systems:** ECM diffusion and permeability has been studied by several researchers using a variety of different techniques applied both in vitro and in vivo, on both cultured and excised tissues. A subset of the results are shown in Table 5.1, as well as Figure 5.4. Fox and Wayland made the first in vivo measurements of tumor diffusion using FITC-dextran and rat serum albumin diffusing in the rat mesentery (Fox and Wayland, 1979). Since that time, Rakesh Jain’s research group has been the field’s major player, authoring one of the original, comprehensive manuscripts on mass transport in tumors (Jain et al., 1980). In 1984 Nugent and Jain introduced a simple fit to diffusion data col-
Figure 5.3: Scanning electron microscopy series showing the variety of pore dimensions in a Matrigel sample. The sample is first fixed using glutaraldehyde, then critical point dried to preserve the gel’s matrix structure.

lected in normal and neoplastic tissues, relating diffusion coefficients of dextran
with molecular weight: \( D = a(M_w)^b \), reporting \( a = 10^6, b = -2.96 \) for normal tissue and \( a = 2.51 \times 10^{-2}, b = -1.14 \) for tumor tissue (Nugent and Jain, 1984). Over the course of the next several decades biologically relevant diffusion experiments have been performed repeatedly by Alan Verkman’s research group (Kao et al., 1993; Periasamy and Verkman, 1998; Lukacs et al., 2000; Verkman, 2002; Papadopoulos et al., 2004; Papadopoulos et al., 2005; Magzoub et al., 2008; Jin et al., 2008; Zador et al., 2008; Zhang and Verkman, 2010), and brain ECM diffusion measurements have been per-
formed extensively by Charles Nicholson’s research group (Nicholson and Sykova, 1998; Nicholson, 2001; Sykova and Nicholson, 2008; Thorne et al., 2008; Hrabetová et al., 2009). Importantly, despite its long-time use as a cell growth medium, Matrigel diffusion has been significantly less studied. Katz et al. performed early experiments on Matrigel hydraulic conductivity, predicting fiber radii $r_f$ (0.626 – 0.696 nm) as well as the void volume ratio $\epsilon$ (0.826 – 0.846) for an unspecified concentration of Matrigel (listed as 8 – 14 mg/ml) (Katz et al., 1992), and later reported $r_f = 3.68$ nm and $\epsilon = 0.87 \pm 0.02$ for 5 mg/ml Matrigel (Katz and Lamarche, 1994).
## Diffusion in ECM - Literature Values

<table>
<thead>
<tr>
<th>Reference</th>
<th>Probe radius (nm)</th>
<th>Matrix</th>
<th>D (x10⁻⁸ cm²/s)</th>
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<tr>
<td>Chary and Jain, PNAS 1989</td>
<td>3.55</td>
<td>mouse tissue</td>
<td>48</td>
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<td>Yuan et al., Cancer Res. 1994</td>
<td>45</td>
<td>human xenograft tumor matrix</td>
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<td>Ramanujan et al., Biophys. J. 2002</td>
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<td></td>
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<tr>
<td></td>
<td>1.9</td>
<td>1% collagen gel</td>
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</tr>
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Figure 5.4: Literature reports of diffusion in ECM and ECM-like materials.
5.5 Fluorescence recovery after photobleaching as a method for studying diffusion

5.5.1 FRAP basics

The experimental framework for FRAP was first reported by Axelrod in a 1976 paper (Axelrod et al., 1976). Axelrod collected initial images of a rhodamine 6G-loaded sample prior to any bleaching. An intense laser was focused to bleach a circular subsection of the sample and the fluorescence intensity recovery in the bleached region was then observed. Because Axelrod’s bleaching experiment resulting in a completely bleached column of material, his fluorescence recovery is ascribed exclusively to lateral transport (diffusion and/or flow) of the rhodamine 6G probes. Careful analysis of the recovery in the bleached sample region (region of interest, or ROI) yielded the absolute mobility coefficient of the fluorescence species, as well as the fraction of probes which are mobile (the mobile fraction). This original paper describes both practical guidelines for FRAP experiments, as well as the theoretical basis for the analysis of FRAP data. Fluorescence microscope technology has come a long way since the mid 1970s, however, and most impressively, the original analysis techniques used to analyze FRAP data are still frequently used some 35 years later (Greene et al., 2008).

Since Axelrod’s original work, the technique of FRAP has been applied to an expansive collection of materials, both organic and inorganic. FRAP has been used
in fields ranging from transport in the cell membrane (Liebman and Entine, 1974; Poo and Cone, 1974) to diffusion on lipid bilayer / carbon nanotube hybrid field effect transistors (Zhou et al., 2007). This largely stems from the simplicity of the technique, as well as it’s ability to provide two key pieces of information regarding the fluorescent probes in the sample. The two most fundamental system parameters determined from FRAP experiments are (1) the diffusion coefficient, $D$, of the probe species (molecules, fluorescent nano/micro-particles) and (2) the immobile fraction of the probe species.

### 5.5.2 FRAP applied to tissues and ECMs

Several researchers have utilized FRAP as a means of studying transport in tissue and ECM. Leddy et al. used the technique to establish diffusion coefficients as a function of depth in articular cartilage (Leddy and Guilak, 2008), as well as diffusional anisotropy in collagen-rich tissues (Leddy et al., 2006); additionally, Fetter et al. used FRAP to probe transport differences between human ankle and knee cartilage (Fetter et al., 2006). Others have used FRAP to study diffusion in pig articular cartilage under various loading conditions (Greene et al., 2008). The most common use of FRAP is in characterizing diffusion rates for cell membrane and intracellular processes. The breadth and frequency with which FRAP has been utilized as a means of characterizing biological materials serves as a validation of the technique’s ability to produce excellent results.

More specifically, FRAP has been used to study diffusion in the ECM. These stud-
ies are aimed at elucidating the transport properties of the ECM at length scales on the order of tens of nanometers, and have been performed in vitro and in vivo by various groups (see references in Section 5.4, 'Macromolecule transport in extracellular matrix systems' subsection, as well as Figure 5.4).

5.6 Experimental work using FRAP

5.6.1 Materials and methods for FRAP experiments

I performed FRAP experiments using a confocal laser scanning microscope (CLSM), which allowed for rapid and highly adjustable bleaching periods, as well as flexible dimensions for region of interest. Throughout this section I’ll be discussing experimental results taken on a Zeiss 510 confocal laser scanning microscope; all data was collected using a 63x, 1.4 NA Plan-Apochromat oil objective. Data is collected on a region of interest (ROI) 512 pixels x 128 pixels, and the beam is rastered in a serpentine manner so as to maximize the frame rate. A circular bleach spot is selected and the average fluorescence intensity, \( f(t) \) in that bleach region of interest (ROI\textsubscript{bleach}) is observed. This fluorescence intensity is normalized by the average fluorescence intensity of area surrounding ROI\textsubscript{bleach}. This normalizing ROI will be referred to as ROI\textsubscript{norm}. An important assumption of FRAP is that \( f(t) \) is proportional to the concentration of unbleached probes in the region of interest. The average intensity in a given region (over the time course of the experiment) is calculated (for each instance of time) using Zeiss software (LSM 510).
5.6.2 Diffusion of dextran molecules in buffer

FRAP calibration data was collected on thin samples (< 14 µm thick); sample thickness was validated before each experiment and bleaching was verified to encompass the entire thickness of the sample, allowing for all diffusion results to be considered using the two-dimensional assumption. Briefly, a thin layer of vacuum grease was spread around the outside perimeter of a 22 x 22 mm² # 1.5 glass slide. FITC-dextran molecules were mixed with phosphate buffered saline (1x) at 1mg/ml and 0.5 µl of the solution was deposited onto the glass slide. A 3 in. x 1 in. glass slide was then placed on top of the 22 x 22 mm² slide and lightly pressed together. Samples were then placed on the microscope for FRAP experimentation. All experiments
were carried out at 23° C.

Fluorescence intensity for ROI_{bleach} and ROI_{norm} are collected, ROI_{bleach} is normalized by ROI_{norm}, and the data is fit to the FRAP equation for 2D diffusion,

\[ D = \frac{\omega^2}{4\tau} \gamma_D = \frac{\omega^2}{2.76\tau_{1/2}^2} \gamma_D \]  

(5.12)

Figure 5.6: This figure shows my data for diffusion in buffer (filled green circles), as well as a few other reports for diffusion in buffer. The black line represents the theoretical values for diffusion based on the Stokes-Einstein relationship. All values represent data collected in PBS.

where \( \omega \) is the radius of the bleach spot, \( \tau \) is the characteristic time, \( \tau_{1/2} \) is the characteristic half-time, and \( \gamma_D \) is 1.0. Calibration results are shown in Figure 5.6 and represent a good fit with theory (Stokes-Einstein relationship), as well as strong agreement with previously reported data for FRAP experiments involving FITC-dextrans in buffer, thus serving as experimental quality control and validation for
the experiments discussed in the following section.

5.6.3 Diffusion of dextran molecules in Matrigel

Similarly, experiments were performed using dextrans (40kDa–2000kDa) dispersed in 8.75 mg/ml Matrigel. These experiments followed a protocol similar to that used in the previous section, however, due to the need to activate the lateral crosslinks present in Matrigel, the films were incubated at 37° C for 20 minutes prior to photobleaching.

![Graph showing FRAP recovery curves for dextran diffusion in Matrigel.](image)

Figure 5.7: FRAP recovery curves shown for the first 60 seconds of a bleaching experiment. These curves are shown for dextran diffusion in Matrigel, and show a decreasing rate of recovery for larger dextrans.

The recovery curves for dextran diffusion in Matrigel are shown in Figure 5.7.
Figure 5.8: FRAP data in buffer and Matrigel. The nanometer dimensions (hydrodynamic diameter) are indicated on the figure in units of nm.

5.6.4 Buffer and Matrigel diffusion results

The FRAP data from experiments in both PBS and 100% Matrigel indicate that experimental parameters are sufficient to obtain reliable results, however what do these values of $D_{gel}$ and $D_0$ mean? Quantitatively, there are several models which allow us to make statements about the gel using nothing more than the quantified diffusion suppression and the radius of the probe. Here I’ll use some of the data reported in the previous sections to calculate various matrix quantities.
Figure 5.9: Fitting the diffusion in Matrigel with the power law equation, \( D = a(M_w)^b \) (Nugent and Jain, 1984), we found \( a = 295.78 \) and \( b = -0.6593 \).
5.7 Results and Discussion

In the first two sections of this chapter I motivated the study of transport through the ECM and introduced the structure and function of the ECM. Section 5.4 focused on diffusion in biological polymers, including a few of the basic models useful for studying diffusion suppression in these materials. Through these models I hinted at the length scales of pores, probes, and fibers. Additionally, the parameters of volume fraction and $D_{gel}/D_0$ were introduced and their relationship was observed via the various models being used. Sections 5.4 and 5.5 were spent introducing FRAP as an experimental procedure and discussing the FRAP data collected, including experimental values collected for $D_{gel}/D_0$. In this section I will take these values and view them in light of the Renkin, Ogston, and Phillips models, as well as discuss what $D_{gel}/D_0$ indicates about the tortuosity of Matrigel.

**Renkin model:** Renkin’s model directly relates $D_{gel}/D_0$ with $r_s/r_p$; here it will be used to estimate the pore dimension in our samples, since $D_{gel}/D_0$ and $r_s$ are known. The resulting pore radius predictions using the Renkin model are (using the $r_s$ for 40kDa, 70kDa, 250kDa, 500kDa, 2000kDa dextrans, in that order): 42.8nm, 45.4nm, 30.8nm, 50.3nm, and 84.3nm. This data (aside from the 250kDa dextran) points to the notion that larger probes, on average, “see” larger pores, due to their simply being sterically excluded from regions of the sample containing pores smaller than their diameter. Close examination of the SEM image shown in Figure 5.3 clearly indicates that there are matrix pores for which $3.5\text{ nm} < r_p < 24.6\text{ nm}$ (i.e., there are
clearly pores with pore radii $r_p$ smaller than 25 nm).

**Ogston model:** The Ogston model, while ignoring hydrodynamic effects, can still provide for us a loose approximation for the relevant values of $r_f$ and $\phi$. Reports indicating $r_f$ values ranging from 0.7 nm up to several nanometers have made it clear that there is no one exact value which can be accurately and repeatedly used for the Matrigel $r_f$. Therefore, in obtaining the general shape of the Ogston $D_{gel}/D_0$ values over a range of volume fractions, I have chosen to plot how volume fraction would predict $D_{gel}/D_0$ for the largest and smallest dextrans used, *assuming a range of fixed $r_f$ values* (Figure 5.10).

![Figure 5.10: This is the Ogston model prediction for $D_{gel}/D_0$ for a range of volume fractions, given the listed solute and fiber radii.](image)

These predictions based on known $r_s$ values and presumed $\phi$, $r_f$ values, can be compared with experimental results, as the experimental data has been collected for this comparison. In Figure 5.11 I use the $D_{gel}/D_0$ data for each dextran $r_s$ to predict $r_f$
for the entire range of $0.001 < \phi < 1$.

Figure 5.11: Image showing the predicted values for fiber radius as a function of an assumed volume fraction, based on the Ogston model equation. Fissell et al. (Fissell et al., 2009) presented permeability data which indicates an approximate volume fraction for Matrigel of 0.54 (black dashed line on figure). Using this volume fraction, my FRAP data would predict a fiber radius between 7 nm and 12 nm. This differs from Fissell’s simulation data by a factor of 2-3 (Fissell et al. produced a model which predicts a fiber radius of 4.2 nm)

Phillips model: Because the Phillips model can be easily separated into steric and hydrodynamic components, it is helpful to plot the theoretical diffusion suppression as a function of volume fraction. Using each of these terms separately and inserting what we know about the solute radii we can extract expectations for the fiber radius
and obtain the theoretical relationship (across a wide span of volume fraction) for $D_{\text{gel}}/D_0$. This is done in Figures 5.12 and 5.13. Figure 5.14 combines both $F$ and $S(f)$ from the Phillips model, and indicates a significant overprediction for diffusion suppression (Phillips model predicts a lower $D_{\text{gel}}/D_0$ than is experimentally observed). This has been noted in other biological polymer diffusion experiments (Spero, 2010).

![Graph showing diffusion suppression](image)

**Figure 5.12**: This figure shows the prediction for diffusion suppression for a given volume fraction of fibers, when only the steric factor of the Phillips model is employed. The assumed solute and fiber radii for each condition are shown at the right.

Through Figure 5.12 it becomes clear that, using only the steric factor $F$, looking between $0.1 < \phi < 1$ and observing probes with $r_s$ or 24.6 nm, fiber radii of 0.5, 1, 2, and 5 nm all result in $D_{\text{gel}}/D_0$ values which at or very near to 0. This indicates that if the fiber radii were in this range, the largest dextran molecules actually would not diffuse much at all (they would fall into the category of probes being much larger than the average pore dimension). This, we know from the FRAP experiments, is
not the case. Interestingly, using $F$ also predicts $D_{gel}/D_0 \sim 0$ for 3.5 nm probes in the range of $0.1 < \phi < 1$.

Looking at the hydrodynamic effects through the lens of the Phillips model makes obvious a major qualitative difference between hydrodynamic and steric effects: as may be expected, steric effects are significantly more impacted by factors such as probe dimension and fiber dimension. Figure 5.13 demonstrates this, as the predicted $D_{gel}/D_0$ lay significantly closer to one another compared to the steric portion of the Phillips model. This is as expected, and demonstrates that $S(f)$ only modifies the more significant steric component of the Phillips model.

A comment on the hydrodynamic drag effect in ECM: It should be noted that while the pure Ogston model prediction and steric component of the Phillips model predict conceivable values for diffusion suppression in ECM gels, when steric and
hydrodynamic effects are combined using the full Phillips model it becomes apparent that $FS(f)$ severely underestimates $D_{gel}/D_0$. This is consistent with previous reports of underestimating $D_{gel}/D_0$ in polyacrylamide (Tong and Anderson, 1996; Park et al., 1990), alginate (Amsden, 1998), and carrageenan gels (Johansson and Lofroth, 1993; Johansson et al., 1993). Phillips mentions that this underprediction may be due to the fact that the model uses a homogenous matrix with rigid fibers which are tightly fixed in location. In actuality, and particularly in biological polymer materials, the matrix is inhomogenous and contains flexible fibers which are not tightly fixed in space but may actually exhibit some self-diffusion, depending on how the matrix is assembled. Approximately 20% of Matrigel proteins are not incorporated into the matrix and are therefore available for free diffusion; this is another aspect of the gel which is not captured in the models for steric and hydrodynamic hinderance. Additionally,
the assumption in each of these models is that the probe is perfectly spherical in shape. These experiments employ dextran molecules due to their non-adhesive properties with respect to proteins, as well as the their availability in a wide range of sizes. However, the dextran molecule is not spherical. Here the radius of hydration has been used to approximate the dextrans as spherical. This approximation seems reasonable, as the dextrans exhibit the expected sphere-based diffusion coefficients when placed in buffer. Even still, some researchers have proposed enhanced diffusion coefficients of dextrans in extracellular matrix materials due to dextran transport via reptation through matrix pores (Alexandrakis et al., 2004).

5.7.1 Assessing the models

Naturally, there are strengths and weaknesses of the models used here. The Renkin model is perhaps the most simple. Assuming knowledge of $D_0$ and $R_H$, an experiment determining $D_{gel}$ will enable the determination of $r_p$, or vice versa. This model is somewhat set apart from the Ogston and Phillips model, as it uses the pore radius $r_p$ parameter as opposed to the fiber radius $r_f$ parameter. This is significant: if the user is most interested in the actual dimensions of the matrix pores, on average, and is indifferent to the parameter of volume fraction $\phi$ or fiber radius $r_f$, then this model is ideal. Renkin’s model is also set apart in how it envisions the relevant space: while Renkin’s model assumes the material to be a solid with open pores, Ogston and Phillips both consider the material to be open space with cylindrical struts or fibers oriented in that space. As will be shown in Chapter 6, this model seems
to have predicted a reasonable range for pore dimensions: 200 nm diameter rods will be shown to exhibit markedly different behavior compared to 18 nm diameter rods when undergoing magnetophoresis in Matrigel (albeit, the experiments are performed using different Matrigel concentrations). The significance and strength of the Renkin model compared to the Ogston and Phillips models is this: if the question at hand is simply, “Is my particle in question much smaller than, on the order of, or much larger than the pore dimensions of the matrix I am interested in studying?”, then the Renkin model is an efficient way of informing this question. Of course, it is not any help in understanding the fiber dimensions of the matrix being studied. Obtaining an understanding of $r_f$ requires another model altogether.

Both the Ogston and the Phillips models are capable of informing a study regarding the matrix fiber radius, however say nothing about the pore dimensions of the matrix. Instead, they utilize the volume fraction $\phi$ as an indication of essentially how crowded the volume is with space-occluding components – the protein fibrous matrix in this case. While Renkin’s model uses only $D_{gel}$ and $r_p$ as essentially unknown variables, both the Ogston model and the Phillips models have three variables which can be tested: volume fraction $\phi$, diffusion in the gel $D_{gel}$, and fiber radius $r_f$. In this sense, the Renkin model more readily provides an estimate of a matrix parameter with minimal effort. The Ogston model has an advantage in that it is the most simple model for analysis of the relationship between $\phi$ and $r_f$, as knowledge of $D_{gel}$ allows one to plot, over the range of reasonable volume fractions. In many cases, this can be reasonably estimated. For synthetic samples for which the involved molecules
and exact concentrations are known, $\phi$ can be calculated and, from this combined with $D_{gel}$, the effective fiber dimension can be calculated. Of course, with its ease of calculation comes the cost of ignoring hydrodynamic drag effects. Using the same data and a few more calculation steps, the Phillips model allows for a more inclusive estimation of $r_f$. However, with this comes the likelihood of overestimating drag for the case being studied, as has been described by others (Park et al., 1990; Amsden, 1998; Johansson and Lofroth, 1993; Johansson et al., 1993; Tong and Anderson, 1996). This is the primary disadvantage of using the Phillips model. Based on the results from this chapter and utilizing Fissel’s estimation of a Matrigel volume fraction of $\approx 0.54$, the Ogston model appears to be the better estimate of matrix parameters (due to the severe underprediction of $D_{gel}/D_0$ obtained using the Phillips model).

Of course, there is also the electron microscopy data to take into consideration. While this data points to a fiber radius of $\sim 5$ nm, the data is primarily composed of collagen and laminin images. This data represents the fiber dimensions as seen by an electron microscope. While this is a useful parameter, it does not truly reflect the perspective we are interested in quantifying. This value is better defined by the question: What fiber dimensions do the particles feel like they are traveling through? This is what I have set out to answer in this chapter.
5.8 Conclusions and contributions

In this chapter diffusive transport through Matrigel has been discussed and the relevant models introduced; the structure and function of the ECM was described, as well as FRAP experiments elucidating size-dependent diffusion in Matrigel. Using the proposed relationship between $D_{gel}$ and dextran $M_w$ (namely, $D_{gel} = a(M_w)^b$), $a$ and $b$ were determined for the first time in Matrigel (Figure 5.9). The Renkin model was used to make predictions regarding pore dimensions, while the Ogston model was used to predict fiber dimensions and volume fraction. The Phillips model was used, first by applying only one component at a time (either steric or hydrodynamic drag component), and finally by combining both terms. The significance of collecting diffusion data over a large range of $r_f$ values was elaborated upon, and using this data macromolecule diffusion was modeled using an equation previously developed by Nugent and Jain for predicting macromolecule diffusion in excised ECM. The method of comparing models and model-predicted values demonstrated in this chapter offers a novel perspective for understanding Matrigel as a material. While passive transport (diffusion) in Matrigel has been the primary concern of this chapter, magnetophoretic applications of nanoparticles are the main subject of this research. As such, the next chapter deals with single particle magnetofection through Matrigel.
Chapter 6

Magnetophoresis of Nickel Nanorods in Matrigel

6.1 Introduction

In Chapter 2 I introduced some of the advantages of using magnetic force to enhance transport in various drug / DNA / therapeutic delivery processes. In Chapter 3 I quantified the magnetic properties of Post-Particles by applying forces to these particles and measuring their translational motion in a fluid of known viscosity, under a known field and field gradient. Their transport properties were used to calculate magnetic properties of a single particle ($\chi, M$), via the relationship between the applied force and the drag force on the particle – that is, the Stokes equation. Chapter 4 introduced a process for making magnetic particles via templated electrodeposition, fabricating Janus magnetic nanorods, and making use of a rotational manipulation mechanism for inducing directed rod transport at the floor of a fluidic
chamber. Chapter 5 delved into the structure of the extracellular matrix Matrigel and the characteristics and size dependence of macromolecule diffusion in this material.

Biodistribution of nanoparticles is at least partially dictated by the physics of mass transport. In Chapter 5 I discussed one component of that field, diffusive transport, and commented on diffusion in a biopolymer material as a function of probe size. Importantly, the data collected in Chapter 5 is essentially macroscopic data: spatially speaking, it is obtained from a wide area and analysis indiscriminately includes the diffusion of thousands of dextran molecules collectively. That is the nature of FRAP, as performed in the previous chapter. Here it is important to recall a significant approximation made for the data in the previous chapter, namely, that the ECM is spatially homogenous. While synthetic matrix materials have the potential to be spatially homogenous, this is not actually the case for ECM. This chapter explores why that is important for the process of magnetofection.

In this chapter I examine the transport characteristics of magnetic nanorods undergoing magnetophoresis in Matrigel. By observing and tracking individual rods over long timescales (thousands of seconds) I observe differences in particle transport as a function of rod diameter. As the data for this analysis is performed over the course of thousands or tens of thousands of seconds, these experiments successfully analyze individual particle behavior over time courses relevant to actual in vivo drug delivery. Specifically, in this chapter I observe the particles through several different perspectives. The magnetic force perspective allows comparison between applied force and transport velocity, while the apparent viscosity perspective allows
me to interpret the apparent viscosity of the surrounding medium based on the relationship between applied force and observed particle velocity. It should be noted that different diameter rods experience the same Matrigel environment as drastically different apparent viscosity environments due to their varying degrees of interaction with the protein meshwork of the gel. I will focus on the magnetic fields and field gradients necessary to pull particles of various diameters through Matrigel, quantifying the required force for transport. In this chapter I also use the nanoparticle drug delivery perspective, assuming either volume-loading or surface-loading of a relevant therapeutic. As such, I take the perspective of delivering drugs on a per time basis, and compare the various particles used via this perspective. This drug delivery figure of merit approach provides the somewhat abstract notion of particle velocities and apparent viscosities with a very tangible measure capable of answering the basic question of “How much drug can one move per particle for a given rod type?” This chapter

- demonstrates the first single particle tracking experiments on variously sized nanorods,
- demonstrates the first observation and quantification of single particle fits-and-starts motion for rods undergoing magnetophoresis, and
- demonstrates the first effective viscosity calculations for nanorods undergoing magnetophoresis in Matrigel.
6.1.1 Magnetic drug targeting: early clinical trials

To date, there have only been three clinical trials testing the feasibility of using magnetic drug targeting. Original experiments were performed by Lübbe et al. and involved delivering ferrofluid-bound epidoxorubicin to 14 patients with advanced solid tumors (Lübbe et al., 1996). Of the fourteen patients, three received one course of MDT treatment, nine received two courses, and two received three courses (due to positive responses after the first two courses). The patients had a variety of cancer types, including metastatic breast cancer, chondrosarcoma, squamous cell sarcoma, and others. All patients had been previously treated, unsuccessfully, with standard chemotherapy. Ferrofluid-bound epirubicin was delivered via i.v. to a vein located contralateral to the tumor, and a magnetic field was set up at the tumor site. The magnets used had field strengths between 0.5 and 0.8 T. Magnetic particle accumulation was determined via magnetic resonance tomography (MRT), and six patients experienced significant accumulation at the tumor sites. As could be expected, unsuccessfully targeted MNPs accumulated in the liver. A second clinical trial involved four patients with inoperable hepatocellular carcinomas. MNP-bound doxorubicin was delivered to the patients’ livers via catheter, the catheter channeling MNPs into an arterial feed to the diseased organ. Once within the organ vicinity, the application of a magnetic field gradient pulled the particles out of the artery and into the organ tissue. All four patients experienced a reduction or stabilization of tumor volume. Interestingly, despite the applied particles ranging in size from 500 nm to 5 µm, no embolization was noticed (Wilson et al., 2004).
The same researchers were involved in a larger, 32-patient trial (phase I/II) of magnetic drug targeting. These tumors were targeted using a similar artery catheterization procedure, and a magnetic field of 500 mT was used for MNP accumulation. Post-treatment MRI indicated that MNP targeting was successful in 30 of the 32 patients. Of the participants, 17 patients’ tumors were analyzed in depth: 15 of the patients exhibited shrinking tumor volumes (Koda et al., 2002). Since these reports, several animal models have been used to study the applicability of MDT and have, generally, met with success, indicating that with further clinical trials and a better
understanding of particle transport within the tumor volume, the technique may serve as a future method for treating localized tumors.

These early demonstrations of MDT success have partially fueled an explosion of research in the field of magnetic nanoparticle synthesis, functionalization, and application, including a wide variety of in vitro and in vivo animal studies. Clearly, the problems involved in making MDT a successful therapy are complex and multi-faceted. As the field moves forward, it has become apparent that there are a variety of hurdles which must be overcome, not the least of which is understanding how particles transport through biomaterials and, as a result, engineering particles for efficient transport. Recent reviews have thoroughly covered the general topic of magnetic drug delivery (Polyak and Friedman, 2009; Dave and Gao, 2009; Veiseh et al., 2010; Ahmad et al., 2010; Prijic and Sersa, 2011; Mahmoudi et al., 2011). While much progress has been made in the field observing agglomerations and moving fronts of particle fields in gels en masse, a quantitative, single particle analysis of transport through ECM has not appeared.

6.1.2 Two methods of applying force for magnetic drug targeting

Moving towards the specifics of MDT, it is relevant to discuss the two different methodologies which exist for applying magnetic force at a given site: external magnet placement and internal magnet placement (generally speaking, permanent magnets have been the primary sources of force in these experiments, however electromagnets with time-varying fields and field gradients have recently been tested in
In the first method, these permanent magnets are placed outside of the body, near the desired site of delivery. This method has the advantage of being simple and non-invasive, however frequently requires that the target site be within a few centimeters of the patient’s skin. The second method involves surgically implanting a small magnet at the desired delivery site, and allowing this implanted magnet to perform the functions of particle capture and concentration. This technique has the advantage of being operable in target regions significantly deeper than the surface-applied magnetic fields, and can theoretically be utilized at any location in the body. Of course, the major drawback to this technique is that it is invasive and requires surgery. Additionally, the position of the magnet in vivo should be monitored to ensure it remains in the desired location over the course of the treatment. Also, the two techniques may be combined for reaching locations intermediate to a surgical implant and the skin (Polyak and Friedman, 2009). Future applications of MDT will likely employ multiple magnetic field sources, arrayed complexly around a patient so as to optimize targeting.

6.1.3 What bearing does this chapter have on magnetic drug targeting?

This chapter brings together many of the concepts covered in Chapters 2 – 5, applying magnetic forces to electrodeposited rods in Matrigel. I discuss magnetically guided particle transport through polymer systems, with an emphasis on the rela-
Figure 6.2: Figure from (Polyak et al., 2008) indicating the potential for multifunctional devices, such as magnetic targeting on-board a stent.

A tumor volume is a complex space for particle transport, and systematic delivery of nanoparticles relies on three distinct steps: (1) blood-borne transport to various regions within the tumor site via the vasculature, (2) nanoparticle transport through the vessel wall, and (3) effective and long range motion through the interstitial matrix space. On one hand, the tumor vasculature is inherently leaky, containing holes through which particles traveling through the vasculature can easily leak out (Hobbs
et al., 1998). Particle leakage reduces the fraction of particles that make it from the blood stream and into the tumor extracellular matrix, and particles smaller than 100 nm in diameter are frequently leaked from tumor vessels via these vasculature holes. On the other hand, the ECM’s dense meshwork of proteins can significantly hinder the diffusive transport of larger particles (Netti et al., 2000; McKee et al., 2006). Recently, Wong et al. proposed a “multistage” drug delivery technique for overcoming this problem, which involved embedding 10 nm particles into the gelatin matrix of 100 nm particles. These multistage particles were then activated using matrix metalloproteinases (MMPs) as an activation method capable of disassembling the 100 nm particle and thereby dispersing the 10 nm particles. Upon dispersal, the 10 nm particles were able to diffuse significantly farther into the matrix than the original 100 nm particle (Wong et al., 2011).

Going back to the three stages of nanoparticle delivery (blood-borne transport in the vasculature, transport through the vessel wall, and transport through the ECM), this chapter demonstrates experimental data on magnetically induced transport for the third step of this process. I use magnetic fields and field gradients approximately 1/3 those used during in vivo experimentation (Lubbe et al., 2001) to pull particles through 4.5 mg/ml Matrigel. Importantly, both the particle sizes and the matrix density used in this study are relevant in vivo. The cadre of potential magnetic drug targeting nanoparticles range in size from ~ 20–200 nm (in this chapter I test Ni rods with diameters as small as 18 nm and as large as 200 nm; while Ni nanomaterials may not be ideal for in vivo applications due to toxicity, these particles are easy
to synthesize, experience less oxidation than their Fe counterparts, and are more chemically and mechanically robust than their Fe counterparts). With respect to the transport medium, what is truly important is the collagen content of the interstitial matrix. For example, the human colon adenocarcinoma LS174T has an IM collagen content of 9 mg/ml (Ramanujan et al., 2002). In this chapter I test a collagen-rich matrix with a density of 4.5 mg/ml, a factor of two less dense than the ECMs of some tumors in vivo. The experimental parameters being approximations for the particles and biopolymer complexes involved during in vivo magnetic drug targeting means that the forces used and resulting transport observed during these experiments have some bearing on the processes that take place during in vivo experimentation. This chapter experimentally demonstrates and analytically quantifies the applied force and resulting transport of nanorods with three different diameters. Perhaps most importantly, the concepts presented regarding the relationships among nanorod size, applied force, and magnetophoretic behavior through protein rich gels are general. While they will scale with various particle sizes, applied forces, and matrix densities, this chapter offers the first quantitative analysis of these parameters based on experimental data using a single particle perspective.
6.2 Magnetophoresis through polymers – recent progress and reports

The significant volume of in vivo work on magnetically-guided drug delivery has resulted in a sub-field of research interested in quantifying particle transport through polymers, the aim being to provide quantitative information regarding the particle-force relationships and to document how particles transport through various biological environments. Then end goal of this sub-field is to achieve a better understanding of the mechanisms involved in magnetophoresis through complex polymer / biopolymer materials. As a brief reminder, the current importance of research in this field was recently validated by Ruenraroengsak et al., embodied in the statement that "A better understanding of the movement of nanoparticles in the complex environments of tissues, tumours, blood and lymph is essential for prediction of their behaviour (Ruenraroengsak et al., 2010).” Here I present, in chronological order, the salient work in the field.

<table>
<thead>
<tr>
<th>First Author</th>
<th>Year</th>
<th>Polymer system</th>
<th>Particle dimensions (diam. in nm)</th>
<th>Single particle tracking?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holligan</td>
<td>2003</td>
<td>0.6% agarose</td>
<td>20</td>
<td>no</td>
</tr>
<tr>
<td>Kalambur</td>
<td>2005</td>
<td>collagen I</td>
<td>10, 50, 2800</td>
<td>no</td>
</tr>
<tr>
<td>Kuhn</td>
<td>2006</td>
<td>Matrigel</td>
<td>270, 290, 800</td>
<td>no</td>
</tr>
<tr>
<td>Kuhn</td>
<td>2006</td>
<td>Matrigel</td>
<td>290</td>
<td>no</td>
</tr>
<tr>
<td>Salloum</td>
<td>2008</td>
<td>agarose gel</td>
<td>10</td>
<td>no</td>
</tr>
<tr>
<td>Basak</td>
<td>2009</td>
<td>0.6% agarose</td>
<td>130, 250, 110</td>
<td>no</td>
</tr>
<tr>
<td>MacDonald</td>
<td>2010</td>
<td>Surgilube gel (15,000 cP)</td>
<td>239</td>
<td>no</td>
</tr>
<tr>
<td>Cribb</td>
<td>2010</td>
<td>DNA solutions</td>
<td>200 (rod diam.), various lengths</td>
<td>yes</td>
</tr>
</tbody>
</table>
6.2.1 Holligan et al., 2003

In this work the authors perform experiments in which approximately spherical particles with 10 nm diameters are injected into a volume of 0.6% agarose, and then pulled through the agarose using a permanent magnetic field applied over the course of three days. The particles are guided towards the magnetic field source, and imaging of the experiment is performed on a macroscopic level. The authors describe the force balance of drag force ($F_{\text{drag}}$) and applied magnetic force ($F_{\text{mag}}$) to obtain an applied force of 0.11 fN for a single particle, and a resulting average velocity of 77 nm s$^{-1}$. Particle transport is observed en masse, looking at dark regions of the agarose gel sample to determine the average particle location (Holligan et al., 2003).

The model used by Holligan et al. employs the viewpoint of hard spheres suspended in a fluid, making use of assumptions of force, viscosity, and solution homogeneity. Here the authors begin with the standard model of a particle moving through a fluid ($v = (1/9 \mu \eta) \chi r^2 \nabla B^2$), and then apply a correction factor based on the knowledge that their fluid is actually composed of a porous, water-filled polymer matrix, meaning that the material actually has some void fraction, denoted as $\varphi$ (for the 0.6% agarose solution used, $\varphi = 0.2$). Thus, the velocity equation is amended to account for this: $v = (1/9 \mu \eta) \varphi \chi r^2 \nabla B^2$. 

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6.2.2 Kalambur et al., 2005

In this paper Kalambur et al. reports on the movement and heating capabilities (for magnetic hyperthermia applications) of 10 nm uncoated Fe$_3$O$_4$ particles, 50 nm dextran-coated γ-Fe$_3$O$_4$ particles, and 2.8 µm uncoated polystyrene beads. The authors report on applying both constant and alternating magnetic fields, and track the leading edge of the nanoparticle collection. The magnetic field is modeled and the distance moved is plotted versus the elapsed time for each of the three particles sizes, noting that the 10 nm uncoated particles covered a 10 mm distance in approximately 1 minute, while the 50 nm dextran coated particles took approximately 11 minutes, and the 2.8 µm beads 12 minutes (Kalambur et al., 2005). The authors note that the slow motion of the larger particles may be due to the larger particles becoming entrapped in the collagen matrix.

Kalambur et al. develop a model beginning with the equations for the magnetic force and the viscous force on a particle in the given scenario. The diffusion force is mentioned, however due to the length scales over which transport is observed (a few millimeters) the authors ignore (probably quite rightly) the significance of the diffusional force in their overall analysis. Both glycerol and collagen solutions were used as transport media. The differences between glycerol and collagen matrices are noted, specifically that collagen is a viscoelastic material with complex rheological properties, and the authors indicate that “Studying the movement of single nanoparticles over length scales of interest in vivo (µm - mm) is experimentally difficult (Kalambur et al., 2005).”
6.2.3 Kuhn et al., 2006a, Kuhn et al. 2006b

These two papers by Kuhn demonstrate the significance of surface functionalization and size with respect to magnetically guided nanoparticle transport through Matrigel. In Kuhn’s Annals of Biomedical Engineering paper the authors demonstrate magnetically guided transport of a magnetic nanoparticle globule which moves through the matrix en masse. Despite being composed of particles 270-800 nm in diameter, the transport is quantified based on the location of the globule which measures several hundred micrometers in diameter. The authors note that both nanoparticle size and surface functionalization have an impact on transport, showing that small particles with PEGylated surfaces transport most effectively, even when transport is based on macro-scale agglomerates of particles (Kuhn et al., 2006b). Kuhn’s Nano Letters paper utilizes the same experimental setup to show that attaching collagenase to the particle surface significantly increases the velocity of the agglomerate (Kuhn et al., 2006a). These two papers serve to significantly inform the field of how particle surface affects magnetically driven transport in a very physiologically relevant polymer system, Matrigel.

One report focuses on the significance of surface functionalization, reporting NP properties, velocities and magnetic field conditions (Kuhn et al., 2006a). However, another of Kuhn’s papers makes use of the previously described methods for implementing magnetic force and drag force for a calculation of the force-velocity relationship. Equating the magnetic force, \( F_M = (\chi_V V_m \nabla B^2)/(2\mu_0) \), and the drag force on a sphere, \( F_D = 6\pi \eta r \nu \), the authors utilize \( \nu = (\chi_V V_m \nabla B^2)/(12\pi \mu_0 \eta r) \). This
work expands upon previous assumptions of single particle transport by estimating particle aggregation sizes and the expected velocities for aggregates of various sizes, and comparing these estimates with experimental data.

6.2.4 Salloum et al., 2008

Interestingly, Salloum’s paper is one of the first in the field to note a major difference between synthetic, transparent porous gels and tumor tissue in vivo – namely, that while the polymer gels used as tissue mimics are homogenous, tumor tissue is highly inhomogenous. This work, however, was performed in gels with various concentrations of agarose. As a common practice in the field is injecting nanoparticles into the centers of bulk volumes of gel or tissue, this research relied on the the shape and volume of the injected bulk to help determine pressure-induced transport properties of these particles in agarose (Salloum et al., 2008). As nanoparticle-induced hyperthermia was the goal of the research, single particle tracking was not performed.

6.2.5 Basak et al., 2009

In Basak’s paper the authors focus on the changing force applied to particles as single spherical particles begin to form chains in a magnetic field. They calculate an average velocity for the particles in a 0.6% agarose gel, and then use this average velocity to estimate the number of particles in a single agglomerate, as well as calculate the effective magnetic force applied to the particles. This is a significant
observation for the field, as every study prior to this had assumed forces were
applied on a per particle basis, without adjusting for particle chaining. As will
become evident, this notion of force applied to an elongated particle as opposed to a
single spherical particle, will be extremely relevant for the experiments shown later
in this chapter (Basak et al., 2009).

Basak et al. propose a theory for estimating particle agglomeration and the
applied force, taking into account particle grouping in a field. Using the observed
NP velocities, the authors calculate the expected effective particle size. Building on
the previous work by Holligan et al. (Holligan et al., 2003) and Kuhn et al. (Kuhn et al.,
2006a; Kuhn et al., 2006b), Basak and colleagues first set out equations for the velocity
of a particle with known magnetic characteristics in a known magnetic field gradient.
Then, citing extrapolations of the Navier-Stokes equation for drag on an isolated
sphere to the case of many spheres lined up in the direction of transport, the authors
provide equations for dealing with such a scenario assuming that the agglomerated
particles travel with a constant velocity. Static light scattering was used to quantify
particle motion, with decreasing scattering intensity indicating diminished particle
concentrations (the particles are seeded at one end of a sample chamber, and as
the magnetophoresis proceeds their concentration at that end, and consequently the
scattering intensity, diminishes over time). Similar experiments were performed using
light microscopy, however single particle analysis was not performed.
6.2.6 MacDonald et al., 2010

The work of MacDonald et al. also focuses on transport through a non-biological polymer gel (Surgilube), with experiments being performed using both DC and AC magnetic force application. The AC fields result in significantly higher particle velocities, and the authors make the argument that the increase in particle velocity is due to the rapid motion of the NPs in the AC field, inducing an apparent viscosity around the particles which is lower than that experienced by the particles in the DC magnetic field (MacDonald et al., 2010).

MacDonald and colleagues assemble an analytical model for the applied force on an individual particle due separately to the DC field and the AC field. The calculated force amounts to 3 fN and 0.03 fN respectively. Although the authors do not consider particle chaining or the potential for non-spherical (elongated) transport dynamics, the major contribution of achieving significant transport velocities using AC fields which supply only a small force (sub 0.1 fN) is explored. In their discussion, the authors invoke Stokes’ Law to explain the relationship between applied force and particle velocity. As particle size remains the same for AC and DC cases, and applied force remains nearly the same for both cases ($\Delta F_{\text{applied}} < 1\%$), the authors suggest that the increased velocity for only a fractionally larger force implies that the apparent viscosity in the system near the particles is diminished. Interestingly, one of the major findings of the research was that increased magnetic particle loadings led to increases in average particle velocity, which may suggest that as MNP loading increased, so did chaining and, consequently, variations in the force applied per
6.2.7 Cribb et al, 2010

The work of Cribb et al. is the most similar to the experiments to be discussed later in this chapter. The authors apply magnetic fields to electrodeposited Ni rods, pulling these rods through both Newtonian solutions as well as non-Newtonian solutions of DNA. Additionally, this research differs from other papers in the field in that it applies single particle tracking to observe individual particle velocities, as opposed to averaging over leading fronts or magnetic particle agglomerates. Cribb’s work compares the required forces for moving spheres and cylinders through various materials (Cribb et al., 2010).

In this report, Cribb and co-workers perform careful rheological characterization of Light Karo Syrup (used as a viscous Newtonian calibration standard) as well as three different concentrations of $\lambda$-DNA ($0.7$, $1.4$, and $2.0$ mg/ml). As $\lambda$-DNA solutions reach overlap concentration at $0.07$ mg/ml, all solutions used constitute biopolymer materials above the critical overlap concentration. The solutions used are viscoelastic. During these experiments single particle tracking was used to determine particle velocities, and it was observed that particles did not exhibit any fits-and-starts styled movement. In their model of particle transport Stokes drag is analyzed and compared for both the spherical and cylindrical geometries. Significantly, from this analysis comes an important equation relating the force on a sphere and a rod (given that the two particles have equivalent volumes and are composed
of the same materials): \( F_{\text{cylinder}}/F_{\text{sphere}} = (8(\mu_r + 2))/3 \). In addition to this relationship, the authors experimentally demonstrate differentially induced shear-thinning in biopolymers during magnetophoresis, a first for the field. The analysis indicates that, while the cylinders used will experience increased drag, the applied force will also be larger than that for a sphere of the same material and volume (see previous equation). Plotting the magnetophoresis results for the Newtonian standard solution (Karo syrup) and normalizing the data by the shape dependent drag coefficient the authors find that the (drag coefficient normalized) velocities for rods and spheres are equivalent. However, in \( \lambda \)-DNA solutions the authors note a power-law dependence for the DNA solution apparent viscosity, pointing to the occurrence of shear thinning. The consequence: for these experiments, the rods proved more effective at transport through DNA solutions. This work lays the groundwork for single particle magnetophoresis experiments and, significantly, indicates that significant phenomenon can be elucidated using this method, as opposed to methods which track agglomerates or the fronts of dense waves of particles.

### 6.3 Physical and practical considerations in nanorod magnetophoresis

Cribb et al. performed the first single particle experiments quantitatively describing the differences between transport of rods and spheres of equal volume in a non-Newtonian solutions, using lambda-phage DNA (\( \lambda \)-DNA) as a model sys-
tem. These results indicate that, in \( \lambda \)-DNA, experimentally determined nanorod velocities are 100 times larger than would be predicted by the solution’s zero-shear viscosity. Cribb’s work indicates that, in shear-thinning solutions, the efficacy of magnetophoresis may heavily depend on shape, and that narrow particles with high aspect ratios may induce more shear-thinning and, consequently, may be better at moving through these types of solutions.

In fluids, the force balance \( F_{\text{magnet}} = F_{\text{drag}} \). This regime exists in both Newtonian liquids such as buffer, and non-Newtonian solutions such as the \( \lambda \)-DNA studied by Cribb et al. Within this regime, all particle motion will be continuous for an applied force; that is, the particle will move along at a constant velocity. And for a given field and field gradient, no significant changes in velocity will be observed. However, depending on the rheological properties of the material, the velocity-force relationship may exhibit one of three possible relationships: (1) A linear relationship between particle velocity and applied force is indicative of a Newtonian solution. (2) If linear increases in force produce increasingly rapid particle transport such that the particle has a higher velocity than would be expected for the given force, then the material is experiencing shear thinning. (3) If linear increases in force produce diminishing increases in velocity (velocity increases with increasing force, but at smaller and smaller increments), then the material is experiencing shear thickening. As previously discussed, research to date has reported particle transport through biological materials, including ECM, as having each of these characteristics. While this may appear so macroscopically, on the individual particle level there are hidden
As has been discussed, the ECM is not purely a fluid and, consequently, the force balance of magnetic force and drag force does not persist. Intuitively, it seems reasonable to believe that nanoparticle transport through a complex matrix such as the ECM is not constant, but rather has (1) a distinct high velocity regime, occurring as the particle moves through buffer-filled matrix pores, and (2) a corresponding low velocity regime, occurring when particle motion is obstructed by a dense assemblage of proteins, the immediate local mesh environment having pore dimensions smaller than the presenting dimension of the traveling particle. Yet, to date, no report has demonstrated this bimodal method of translation. In this chapter I report on the first demonstration of combined magnetophoresis and single particle tracking in ECM, and use the data collected to comment on the importance of rod diameter and overall volume to applied magnetic force and transport. Included in this analysis is
the first demonstration of fits-and-starts motion for individual nanorods undergoing magnetophoresis.

6.4 Experimental section

In the next several subsections I discuss experimental details pertinent to carrying out quantitative magnetophoresis experiments via single particle tracking of Ni nanorods in Matrigel. These experiments constitute the first set of single particle tracking experiments performed on rod-shaped particles in a collagen-rich ECM.

6.4.1 Growth and preparation of Ni nanorods

The experiments in this chapter were performed using NRs grown using both Whatman Anodisc 13 AAO templates (pore diameter of 200 nm), as well as Synkera Inc. AAO templates (pore diameters of 18, 55, and 100 nm). Template working electrodes (Ag) were applied via thermal evaporation or sputtering (Kurt Lesker PVD 75). NRs were grown as discussed in Chapter 4; specifically, Ni-Cu multilayers were grown so as to provide the largest number of Ni segments per template. Ag working electrode was etched using HNO₃; the template and Cu segments were simultaneously etched using 3M KOH. NRs were rinsed several times in DI H₂O, followed by rinsing in ethanol. They were stored in ethanol until surface functionalization.
6.4.2 Surface functionalization of electrodeposited Ni rods

Proteins readily adsorb to metal and metal oxide surfaces. Consequently, it is this surface adsorption that determines the properties of the protein-inorganic material complex, as well as how the biological systems in place will respond to the inorganic material (Ratner et al., 1996). The behavior of nanoscale materials is particularly determined by the protein-material relation due to the large surface area to volume ratios inherent in these materials. Overall particle behavior in a protein network is therefore a consequence of composite factors, including steric, hydrodynamic, and chemical interactions. While particles may be small enough to move through the pores of a given protein network or mesh, chemical interactions between the mesh proteins and the particle surface can render the particle immobile. Interactions between metal oxide surfaces and proteins can drive adsorption via several different factors: (1) protein-protein and protein-surface electrostatic interactions, (2) surface or solution dehydration, (3) and protein structural changes can all contribute to protein adsorption (Giacomelli et al., 1997). The metal oxide surface is, natively, covered with a hydroxyl layer. This layer persists in water, as well as when the material is dried and stored at standard temperature and pressure. The hydroxyls in this layer contribute to the formation of the nanoparticle-protein ‘corona,’ the composite of proteins which adsorb to the NP surface for a given setting. Naturally, the protein-surface interaction for metal oxides is not independent of what metal is involved in the oxide and depends substantially on the charge to radius ratio, as well as the coordination number of the metal involved (Fukuzaki et al., 1996).
To ensure that the magnetic force application studies are testing the force-transport relationship independent of protein adsorption, a mediating polymer coating can be used. The notion of using colloidal surface chemistry to moderate or prevent particle-particle interactions has a decades old history (Overbeek, 1977). During the early 1990s several important reports appeared elucidating the protein adsorption properties of surfaces coated with PEG, PEO (poly(ethylene oxide)), or PPO (poly(propylene oxide)) (Gombotz et al., 1991; Prime and Whitesides, 1991; Desai and Hubbell, 1992; Amiji and Park, 1992). These reports paved the way for Winblade et al., who in 2000 used this method of thinking in attaching PEG-based block or graft copolymers to biological surfaces such as cell membranes and tissue surfaces (Winblade et al., 2000) to mediate surface-surface interactions and prevent cell agglutination. A few years later Valentine et al. reported that particle surface chemistry critically affected microrheology results based on multiple particle tracking, as particles which were stuck to the biopolymer network exhibited significantly different diffusive properties relative to particles bearing an adhesion-preventing polymer (PEG) coating (Valentine et al., 2004). Valentine’s research lay the groundwork for later reports indicating that particle transport through biomaterials such as mucus cold be drastically engineered by surface modification with PEG (Lai et al., 2007b; Lai et al., 2007a; Suh et al., 2007; Suh et al., 2007; Wang et al., 2008). Following up on the original work of Lai et al., Wang et al. reported how the mucoadhesivity paradox – the fact that larger beads were observed to show high diffusion suppression when covered in high molecular weight PEG (above 2 kDa) but, but low diffusion suppression when
covered low molecular with PEG (2 kDa or smaller) – may be explained by suggesting that large MW PEGs lead to increasingly complex interpenetrating networks (IPNs) within the macromolecular-biopolymer system. Wang et al. also coated polystyrene particles with 5 kDa PEG and observed that these particles experience small levels of diffusion suppression, just as the 2 kDa PEG coated particles, indicating that the critical molecular weight for the formation of IPNs lies somewhere between 5 and 10 kDa PEG (Wang et al., 2008). In light of this suggestion, the particles used in this experiment were PEGylated using 1 kDa PEG molecules.

In this chapter I use particles functionalized with PEG for all ECM transport experiments. Successful surface modification of electrodeposited Ni micro- and nanorods is achieved via methoxy-PEG-silane chemistry using a previously employed protocol (Zhang et al., 2002). Functionalization is proven via zeta potential measurements and TEM observation of the nanorods before and after PEGylation. Briefly, nanorods are removed from their AAO templates and rinsed eight times in ethanol via sonication and gentle centrifugation. They are then dried at 80°C for 1 hour and vacuum dried overnight to remove adsorbed water. Following drying, the particles are vortexed with 3 mM methoxy-PEG-silane (Laysan Bio, Inc., Arab, Alabama, USA) in toluene and incubated at 60°C for 4 hours. Following incubation, the particles are resuspended via sonication, vacuum dried, rinsed with toluene, sonicated in toluene for 10 min., washed in toluene and ethanol, vacuum dried, and finally resuspended in 1x PBS until experimentation.
Figure 6.4: Native oxide particles and PEGylated particles.

6.4.3 Force application setup

The force application setup is identical to that used in Chapter 3 (force application stage is pictured in Figure 6.6). All pipettes and eppendorf tubes are chilled at 4°C
prior to use, as Matrigel will begin to solidify when it comes into contact with room temperature materials. Sample chambers are created from PDMS and an oxygen plasma is used to activate the PDMS and glass surfaces for sealing. Matrigel is mixed with PBS containing nanorods. The mixture is gently mixed, then deposited into a sample well and gelled at 37°C. Following gelation the sample is placed on the microscope and a sealing layer of mineral oil is deposited on top of the mixture to minimize sample dehydration. The sample is sealed again by placing vacuum grease around the top of the PDMS well and placing a cover slip on top of this. The entire microscope and sample assembly is allowed to settle for approximately three minutes before applying magnetic forces.
Figure 6.6: Stage for applying magnetic forces to rods moving through Matrigel.

6.4.4 Data collection and analysis

The data collection and analysis in this section of the document was performed very similarly to the data collected for Section 3.5 (Post-Particle force calibration).
For these experiments, an inverted microscope with a 100x dry objective was used. Video data was collected using transmitted light and a Pulnix PTM-6710CL camera. Data was collected at 1 frame per second for 36000 s (10 hours) per experiment. After video collection, NRs were tracked using SpotTracker software (Russell Taylor, freely available for download at http://cismm.unc.edu).

6.5 Results and discussion

Just as the ECM is a complex material, magnetically driven transport through this material is also complex. Up until now, reports on magnetophoresis in biological materials have demonstrated constant velocity or, in some cases, an increasing particle velocity in some shear thinning materials (called takeoff). Unique to this chapter is the observation of single nanorods moving through the matrix in fits-and-starts, and a quantification of the velocities and forces involved in the process. No previous reports have observed this type of motion.

6.5.1 Quantifying the force required to move through Matrigel

Chapter 3 made use of Stokes drag to calculate the forces on particles. In this framework, the drag force \( F_d \) and the magnetic force \( F_{\text{magnetic}} \) are equilibrated, as the particle assumes a constant velocity and the net force on the particle is zero. This framework is operational for low Reynolds number systems in which (1) the drag is assumed to be imposed by molecules which are (very) small relative to the
particle being transported, (2) the media is homogeneously distributed (there exists a uniform density throughout the path of particle motion), and (3) all components of the media are allowed to flow around the moving particle – that is, there are no immobile components in the media.

In such an environment, Stokes’ equation can be generalized by \( \vec{F} = \beta \eta \vec{v} \), where \( \vec{F} \) is the force, \( \beta \) is a shape factor, \( \eta \) is the viscosity, and \( \vec{v} \) is the particle velocity. For a sphere, \( \beta_s = 6\pi a \), where \( a \) is the sphere radius. For a cylinder,

\[
\beta_c = \frac{2\pi L}{\ln\left(\frac{L}{r_c}\right) + \gamma_\parallel},
\]

where \( L \) is the cylinder length, \( r_c \) is the cylinder radius, and \( \gamma_\parallel \) is the end correction factor (\( \gamma_\parallel = -0.19 \) for our case) (Tirado and de La Torre, 1979; Cribb, 2010; Cribb et al., 2010).

Due to the immobile meshwork structure of the ECM, the sizes of the proteins involved, the sizes of the NRs being pulled, and the innate heterogeneity of proteins in the ECM the assumptions necessary for implementing Stokes Law cannot be used. Whereas we were previously allowed to implement the Stokes equation in setting \( F_d = F_{\text{magnetic}} \), in the setting of the ECM this no longer holds. As such, we will take an analytical approach to calculating \( F_{\text{magnetic}} \) using the magnetic properties of the NR and the applied field and field gradient to determine the magnetic force.

From previous work (Cribb et al., 2010) the magnetic force applied to a sphere and a prolate ellipsoid can be calculated based on particle volume (\( V_s \) for a sphere,
\[ F_s = \frac{(\mu_r - 1)}{2\mu_0} \frac{3}{8(\mu_r + 2)} \cdot V_s \cdot \nabla(B^2), \] (6.2)

and for an ellipsoid we have

\[ F_c = \frac{(\mu_r - 1)}{2\mu_0} \cdot V_c \cdot \nabla(B^2). \] (6.3)

Based on these relationships we can calculate the forces on a rod of a given dimension. The field of a cylindrical magnet \( B_m(z) \), at the center of the magnet along the magnet axis, is

\[ B_m(z) = \frac{\mu_0 M_0}{2} \left[ \frac{L_m - z}{\sqrt{R_m^2 + (z - L_m)^2}} + \frac{z}{\sqrt{R_m^2 + z^2}} \right], \] (6.4)

where \( L_m \) is the length of the magnet, \( R_m \) is the magnet radius, and \( z \) is the distance from the magnet face (Cribb et al., 2010). The first derivative of this is the field gradient:

\[ \nabla B_m(z) = \frac{\mu_0 M_0}{2} \left[ \frac{-z^2}{(R_m^2 + z^2)^{3/2}} + \frac{1}{(R_m^2 + z^2)^{1/2}} + \frac{(L_m - z)^2}{(R_m^2 + (z - L_m)^2)^{3/2}} + \frac{1}{(R_m^2 + (z - L_m)^2)^{1/2}} \right]. \] (6.5)

These calculations, of course, rely on a magnetic rod which is aligned with the field direction.
This is useful in calculating the field and field gradient experienced by the nanorod. By analytically calculating the force on a rod of known dimensions we can compare how this force scales with rod length for a given rod radius, as well as a sphere of a given radius (assuming all particles have the same composition – nickel in this case). Additionally, we can compare how the application of force on variously sized rods plays out in NR transport.

6.5.2 Rod sizes, forces, and expectations

Using the known rod diameters and measuring the lengths of individual rods being tracked (using ImageJ), the rod volume was approximated and, from that, the expected force on the rod. The first and perhaps most important observation is that there is clearly a pore-size effect: smaller rods experiencing significant less force move with significantly higher velocities through the matrix. This simple fact points to the limitations of using a Stokes-based analysis in the extracellular matrix, and indicates that future studies on magnetophoresis should explicitly take this into account.

A few aspects of the data should be discussed. For example, the data for the 18 nm rods shows relatively large spread in velocity. Video analysis indicates that this is due to the fact that some of these rods spent large amounts of time stuck in the matrix, proving that even rods with sub-20 nm diameters will experience extreme steric hindrance. The velocity spread for both 55 nm and 200 nm rods is considerably smaller due to their unanimous steric hindrance: due to their larger diameters, all of
Figure 6.7: Force versus tracked velocity data for 18, 55, and 200 nm diameter rods. Force values are calculated based on the previously discussed equations...

the larger diameter rods experienced significant steric hindrance.

Also, it is clear from the data that the calculated force for the 200 nm diameter rods covers more than an order of magnitude. This is explained by the fact that tracked rods had wide range of lengths, from sub-micrometer (force on the order of 7 pN) to approximately 30 µm (force on the order of 300 pN).

While it is useful to elucidate transport differences between nanorods of different dimensions, this information is perhaps better interpreted using a figure of merit which involves some contribution from the "theoretical drug carrying capacity" of the rod. The notion of a carrying capacity is useful so as to determine if there is any
real benefit from using rods of various sizes: if, after accounting for the theoretical drug carrying capacity, the rods end up moving the same amount of a drug per unit time, then their various velocities becomes a moot point.

There are two basic ways of calculating carrying capacity. In nanoparticle drug delivery, the relevant molecules can either be incorporated into the volume of the carrier particle, or attached to the surface of the NP carrier. So velocities will be normalized once using the particle volume in the figure of volume-normalized merit calculation, and once using the particle surface area for the surfaces area normalized figure of merit calculation.

![Graph showing velocity/volume values for different diameters](image)

Figure 6.8: A figure of merit for the volume-normalized velocity of the rods.

These figures of merit indicate that indeed there are significant implications for the single particle theoretical carrying capacity of these rods based on the volume-normalized velocities. For volume-loading of drugs, Figure 6.8 indicates that the 18 nm diameter rods will have velocity / volume values approximately three orders of magnitude larger than their 55 nm counterparts, and approximately four orders
of magnitude larger than 200 nm diameter nanorods. Assuming the drug is being carried via the particle surface, Figure 6.9 indicates approximately a two orders of magnitude advantage of 55 nm diameter rods over 200 nm, and a two orders of magnitude advantage of the 18 nm diameter rods over their 55 nm counterparts.

The apparent viscosity perspective: Although the experiment and ECM components do not lend themselves to utilizing the relationship $F_{\text{drag}} = F_{\text{magnetic}}$, it is useful to imagine the relevant, calculated value of $\eta_{\text{apparent}}$, which takes into account the probe geometry, applied force, and resulting velocity and calculates apparent values of viscosity. This allows us to interpret the nanorod transport as though it were taking place through a Stokesian medium, and compare how that medium may appear to rods of different diameters. Thus, plotting applied force vs. apparent viscosity shows that the 55 nm and 200 nm diameter rods experience a $\eta_{\text{apparent}}$ values approximately two and four orders of magnitude larger, respectively, than the $\eta_{\text{apparent}}$ experienced by the 18 nm rods.

Figure 6.9: A figure of merit for the surface area-normalized velocity of the rods.
Using this perspective it can be is useful to place our rods in various Newtonian liquids, and, using the analytical method of obtaining applied force, evaluate how we may expect these rods to behave in Newtonian standards of various viscosities. Taking that approach, we can perform the thought experiment of placing our rods in water (1 mPa·s), honey (10 Pa·s), mustard (70 Pa·s), and vegetable shortening (1200 Pa·s). Using assumed viscosities of these various materials, we can assess how quickly our rods will move. From plotting this data we observe a few interesting characteristics. First of all, as increasingly long varieties of a given rod diameter are
pulled in our previously defined field and field gradient, the velocity slowly falls off. However, more importantly, it becomes clear how the Stokes Law prediction differs from the experimental data: while Stokes Law demonstrates ever-increasing velocities for increasing applied force, our experimental data clearly shows a decrease in velocity for increasing applied force, due to the size exclusion effect of the matrix with respect to the larger diameter rods. This demonstrates that of particle shape and diameter are considerably more important than the applied force for some types of transport.

Figure 6.11: Applied force vs. velocity in the experimental data (squares) and in theoretical Newtonian standards (lines). $\eta_{\text{water}} = 0.001 \text{ Pa}\cdot\text{s}$, $\eta_{\text{honey}} = 10 \text{ Pa}\cdot\text{s}$, $\eta_{\text{mustard}} = 70 \text{ Pa}\cdot\text{s}$, and $\eta_{\text{vegetable shortening}} = 1200 \text{ Pa}\cdot\text{s}$. 

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6.5.3 Observations during nanorod magnetophoresis: continuous motion and fits-and-starts motion

During experimentation two distinct modes of transport were observed. The first mode, continuous motion mode, was characterized by rods moving at constant velocities. The second mode, fits-and-starts mode, was manifest by particles moving (forward) quickly some times, and very slowly or not at all (at least not in the forwards direction) during other times (here ‘forwards’ is defined as towards the magnet, or in the direction of increasing field gradient).

**Continuous motion:** In all experiments, 200 nm diameter rods moved exclusively via the continuous motion mode of translation. Interestingly, not only did individual rods move in continuous mode, but also rods within a single sample well seemed to move at very similar velocities. This observation indicates that these rods, instead of moving through the matrix, are actually deforming the matrix as they traveled. There are two perspectives one can take when considering this type of motion.

The first is the macroscopic perspective, considering the ECM-particle sample volume as a single entity, a composite material. This composite material is essentially composed of three components: magnetic rods, buffer, and the ECM protein meshwork. Taking the composite material perspective we can assume a uniform distribution of all three components. However, upon applying a magnetic force, it becomes apparent that the rods act as internal point sources of an internally applied force. However, because the observed motion for this size of rod indicates that the
rods are deforming the matrix as opposed to moving through the pores of the matrix, these point sources can be assumed as acting on the ECM protein mesh component of the composite material, deforming it and pulling the matrix along with them as they translate. Over the course of time this results in a spatial density separation within the composite, with rods and protein meshwork materials moving in the direction of increasing magnetic field gradient, while buffer and more mobile ECM component molecules move in the direction of decreasing field gradient (this, of course, makes use of the fact that the composite material is confined on all sides and, therefore, cannot actually translate in space, but can experience internal component reordering). Evidence for this lies in the fact that no 200 nm diameter rods were observed to be moving consistently or appreciably in the direction of decreasing field gradient, indicating an internal component reordering must be taking place, at the very least with respect to the metal nanomaterial components of the composite.

The second perspective for considering large-diameter rod transport is the microscopic, single particle perspective. While previous experiments commented on the nature of this perspective, these single particle tracking experiments provide evidence as to differences in how this perspective operates for large diameter and small diameter rods. Of course, the most significant observational difference is that small diameter rods move in a fits-and-starts type motion indicative of traveling through the interstices of a porous matrix, while large-diameter rods demonstrate constant velocity motion, indicating that the matrix pores are not actually being probed by the large diameter rods. Why is this significant at the single particle level? Because,
as hinted at in the discussion from the macroscopic composite material perspective, this indicates a compositional reordering. And at the single particle level, this looks like ECM (mechanical) yield. This is an important feature of the microscopic perspective: it allows us to interpret the nanorod transport in terms of mechanical pressure, the force divided by the area. Single particle tracking and the knowledge of the rod dimensions allows for a simple computation of the applied force for each rod. Because the rod has a fixed diameter and applies the calculated force over the resulting circular area, it becomes easy to calculate an applied pressure in terms of pascals: \(1 \text{ Pa} = 1 \text{ N/m}^2\). For the 200 nm diameter rods, the area is \(3.14 \times 10^{-2} \mu\text{m}^2\). The applied force ranges from 6.3 pN to 238.2 pN, resulting in a range of applied pressure from 201 to 7586 Pa. While the applied pressure is straightforward, converting this to a modulus \((E)\) is not. This is because common calculation of \(E\) requires some knowledge of \(\Delta L/L_0\).

**Fits-and-starts motion:** While all 200 nm diameter rods demonstrated constant velocity motion, 55 nm diameter rods displayed a mix of constant velocity as well as fits-and-starts style motion. The 18 nm diameter rods moved exclusively via fits-and-starts type motion, shown here. It should be noted that some of the 18 nm rods spent long amounts of time in ‘traps,’ the longest observed time being approximately 5 minutes. Of course, many traps were significantly more easily escaped, retaining the nanorods for only 2 - 20 seconds. In addition to experiencing fits-and-starts motion, the 18 nm rods also exhibited larger off-axis variation in their transport. While the 200 nm rods traveled consistently along the axis of the applied magnetic force (x-
axis), the 18 nm rods moved along this axis while shifting dramatically (several rod diameters) in the y-axis. Also, while 200 nm rods were never observed to move along the x-axis away from the magnet (they always moved forwards), the 18 nm rods, when interacting with the fibers of a protein matrix trap, were observed to move both forwards and backwards (positive and negative x directions). I speculate that this motion is due to the fibers of the matrix sterically hindering the nanorod’s motion pushing back on the rod in response to its applied positive x-direction force. This does not occur with the larger rods due to the fact that they exert more than an order of magnitude more force on the matrix fibers.

For these rods, the observed motion requires a more careful consideration of the relevant forces involved. Naturally, both large diameter rods and small diameter rods experience magnetic forces and diffusive forces. However, the data indicates that for large diameter rods magnetic forces dwarf diffusive forces, while for small diameter rods these forces are more comparable. Figure 6.12 compares MSD values for each diameter of rod: \( \text{MSD}_{55 \text{nm}} \approx 5 \times \text{MSD}_{200 \text{nm}} \), and \( \text{MSD}_{18 \text{nm}} \approx 20 \times \text{MSD}_{200 \text{nm}} \).

A brief note on rotational diffusion: Clearly, during rod magnetophoresis experiments the rotational diffusion of the translating rod is significantly suppressed due to the torque applied to the rod via the magnetic field. Of course, the rotational diffusion is also suppressed by the rod’s steric interactions with the surrounding ma-
trix. However, our observations indicate that these steric interactions could be easily overcome by the applied magnetic field: randomly arranged rods in the ECM matrix were immediately aligned by the magnetic field, indicating that while steric effects do act on the rods, these forces are significantly smaller than the rotation damping force of the applied magnetic field.

First, it is important to consider a few of the statistical properties of rotational diffusion, denoted by $\theta(t)$. The rotational diffusion of ellipsoidal particles was first
Figure 6.13: (a) Minimum intensity projection of an 18 nm diameter rod moving through ECM via fits-and-starts motion. (b) Overlay of path trace (red) on top of minimum intensity projection. (c) Extracted x-y trace of minimum intensity projection highlighting (arrows) locations at which the rod became entrapped in the matrix and, consequently, spent several seconds (the data is collected at 1 frame per second).

explored by Perrin (Perrin, 1934; Perrin, 1936). The differences between rotational diffusion considerations for spheres and ellipsoids stems from the fact that elongated particles (ellipsoids, cylinders, etc.) have not just one translational hydrodynamic friction coefficient (as is the case for spheres), but rather two: one friction coefficient for motion parallel to the long axis of the ellipsoid, and one friction coefficient for motion perpendicular to the long axis.

As such, it is known that \( \langle [\Delta \theta(t)]^2 \rangle = 2D_\theta t \). Recently, Han et al. performed simulations and corresponding experimental work on ellipsoids, confined to two-
dimensional motion, undergoing Brownian diffusion (Han et al., 2006). In their simulation they establish differences in the expected relationship between $D_t$ (translational diffusion) and $D_\theta$ (rotational diffusion), noting that $\theta$ has a large impact on $D_t$ if the orientation and location are sampled frequently (for example, 10,000 times in their simulation) per $\tau_\theta$, where $\tau_\theta$ is the time it takes for the rod to rotate by 1 radian. However, if the orientation ($\theta(t)$) is sampled with lower frequency (10,000 steps over the course of 100$\tau_\theta$ is sufficient), then the translational diffusion component’s impact on the rotational diffusion is significantly diminished.

What does this mean for our magnetic rods in Matrigel? It allows us a theoretical perspective through which to examine rotational diffusion. Theoretically, the probability distribution function for an ellipsoid diffusion in a Newtonian solution yields a Gaussian distribution for $\Delta \theta(t)$ and a corresponding variance of $2D_\theta t$. Additionally, as demonstrated by Han et al., the angles $\theta(t)$ should be uniformly distributed in the range of $[0, 2\pi]$ radians. As is evident by Figure 6.14, nanorod orientations are not uniformly distributed in the range $[0, 2\pi]$, but are instead clustered around the angle of the magnetic field. Calculating the variance for each rod type results in $\sigma^2_{[200nm]} = 2.8$, $\sigma^2_{[55nm]} = 7.2$, and $\sigma^2_{[18nm]} = 52.3$, demonstrating significantly increased angular fluctuations in smaller diameter rods ($\sigma^2_{[18nm]} \approx 19\sigma^2_{[200nm]}$, and $\sigma^2_{[55nm]} \approx 2.5\sigma^2_{[200nm]}$).
Figure 6.14: A histogram of rod angles with respect to the magnetic field direction (0 degrees). The data demonstrates significantly more variation in the orientation angle $\theta$ for small diameter rods as opposed to larger diameter rods.

### 6.5.4 Potential implications for fits-and-starts styled transport

While the observation of fits-and-starts type motion in magnetophoresis through a biopolymer matrix is no doubt unique, the importance of these observations truly lies in what it tells us about the possibility of engineering systems which more effectively make use of this style of motion. MacDonald’s research presented an argument for local viscosity changes in the vicinity of magnetophoretically actuated nanoparticles in an AC magnetic field (MacDonald et al., 2010), however this fits-and-starts motion offers an entirely new perspective on the types and directions of forces which might serve to increase NP transport in protein rich matrices.

Also, although the particles used in these experiments are rod-shaped, the findings are generally relevant to spherical particles as well. Spherical superparamag-
netic particles will, if near enough to one another, form chains in a magnetic field. Consequently, their motion will be described not by the dynamics of a single particle moving through a gel, but rather by the dynamics of a cylinder or elongated spheroid moving through a gel. This is an important distinction and suggests that perhaps the effects of chaining and the average chain length should be considered relevant when attempting to quantitatively discuss MNP transport in gels.

6.6 Conclusions

This chapter has discussed novel observations of single-particle transport through ECM, noting variations in the transport efficiency as a function of nanorod diameter. The experiments in this chapter demonstrate several firsts:

- the first observation of fits-and-starts motion for sufficiently narrow nanorods;
- a first quantification of velocity vs. force relationship for magnetophoresis involving rods, and descriptions of how force and velocity vary for differently sized nanorods;
- the first figures of merit describing the significance of rod velocities for delivery drugs via volume and surface loading.

From these experiments several aspects of nanoparticle transport through ECM become apparent. Namely, that using the Stokes equation for describing particle motion through a matrix containing meshlike network proteins does not provide a full description of the events occurring during transport, and in fact predicts increasing velocity trends in some scenarios in which experimental data will demonstrate
decreasing velocity trends. Additionally, the observation of fits-and-starts styled motion points towards a transport mechanism which may enable the engineering of force application setups which take advantage of this. For example, perhaps the application of lateral forces (along the y-axis) will allow narrow-diameter rods that find themselves sterically hindered by the local fiber network to escape more quickly, spending less time trapped and more time moving through the matrix pores.

The single-particle perspective presented here is novel, and its significance is compounded by the length of the observations performed. Also novel is the elucidation of drastically different styles of motion for particles of varying diameter, and the implications of these differences on particle velocity. Also new is the observation of increased distribution of angular orientations for small diameter rods versus large diameter rods undergoing magnetophoresis.

Based on these experiments one important conclusion can be drawn: primarily, that particle size and particle force are not equally important throughout the entire range of sizes and forces. In our scenario, small particles experiencing low forces are significantly more effective at moving a relevant drug (for both the volume loading and the surface loading cases, as shown in Figures 6.8 and 6.9)
Chapter 7

Conclusions and Future Directions

7.1 Summary and conclusions

This dissertation has focused on (1) developing techniques for fabricating shape-defined magnetic particles, developing multi-metallic particles via photolithographic methods (Post-Particles), as well as unique magnetic Janus rod-shaped particles, and (2) applying each of these particles to a biologically relevant system. Along the way, a method for quantifying the magnetic characteristics of Post-Particles was determined via quantitative magnetophoresis performed in a known solution viscosity, magnetic field and field gradient. EGFP-correcting antisense oligonucleotides were attached to these particles and, when delivered with a magnetic field, these particles were shown to be useful as magnetofective agents. The fabrication methodology contribution of this work is completed by the creation of Janus-structured rods capable of post-fabrication, side-specific chemical functionalization.

Within the realm of force application, two different types of magnetic fields were implemented for manipulating rods. In a low Reynolds number solution, a rotating
field was used to induce hydrodynamic swimming. The translation mechanism was studied with respect to a relevant force model, with the experimental and theoretical values for translational velocity and angular velocity were shown to be highly correlated. The significance of these experiments is in the ability to maneuver in an arbitrary direction within the two-dimensional plane near the surface, and was demonstrated by using these rods to pickup a bead. These rods were capable of moving beads 133% of their volumes (0.52 $\mu$m$^3$ for beads, 0.39 $\mu$m$^3$ for Janus rods, approximately).

Finally, single particle tracking was applied to magnetically guided motion through the ECM. This single particle tracking elucidated a mode of magnetomotive transport previously undocumented – namely, the ’fits and starts’ motion demonstrated in Chapter 6.

7.2 Future directions

The field of magnetically enabled micro- and nanotechnologies has enormous problems in its sights. As the applications for microfluidic devices leave the laboratory and are integrated into lab-on-chip technologies capable of performing analysis and diagnostic processes outside of the laboratory and quite literally around the world, it is possible that magnetically guided micro-movers will play a role in achieving single-object (or single-cell) manipulation with low power requirements. Additionally, magnetically actuated devices may play a role in on-chip mixing appli-
cations. The rotational manipulation mechanism demonstrated in Chapter 4 holds the potential for performing both manipulation and mixing functions, as their rotation will produce enhanced mixing for some regimes of Reynolds number. Furthermore, and perhaps most ambitiously, scientists and engineers are currently looking to these devices to elucidate mechanisms for swimming and force application in low Reynolds’ number scenarios, with the hope that one day these objects will be able to work alongside the most elegant of movers, protein molecular motors, to perform inter- and intracellular functions.

Additionally, magnetically targeted particles are front-runners for the efficient and localized delivery of medicines, and indeed a number of in vivo studies have been published, with a variety of studies currently underway. Confidence in the field’s enormous potential is demonstrated by Philips’ development of tools designed specifically for this purpose. The work contained in Chapter 6 hopes to inform the field of the relevance of particle size and shape, as well as the accompanying force considerations with respect to transport through extracellular matrix materials. However, the particles used in this section are fairly simple. Other research groups are currently undertaking the fabrication of complex particle shapes. While rod-shaped particles act as an appropriate starting place for elucidating aspects of the shape-transport relationship, magnetic particles shaped as arrows or cones may offer the added benefit of presenting a narrow diameter to the matrix ahead of it, however may also be able to essentially burrow through the matrix. And perhaps, just as macroscopic screw-shaped objects have been used to move through tissue via
essentially drilling through the material, nanoscale screws may be able to perform a similar function at a significantly smaller scale. Chapter 6 argues for the importance of single-particle tracking studies in understanding transport in materials as inhomogeneous as the ECM. It is possible that the methods described in Chapter 3 will be used to create such highly magnetic, arrow- or cone-shaped particles for testing their transport properties through ECM. With reference to the Post-Particles method described in Chapter 3: while the multi-metallic capabilities of the method were demonstrated, the technique was not used to produce particle compositions engineered towards a specific function. Using this technique to create particles with core-shell or Janus structures for binding multiple surface molecule types to a single metal particle is a potential future application of the technique. Additionally, performing various thermal treatments may make possible alloyed particles with specific mechanical properties, and performing various etching treatments may possible particles with tunable porosity, as a metal-specific etchant could be used to selectively remove one species present in the particle. Additionally, the technique could be used to add a Pt component to shape-defined particles for use as catalysis powered motors in \( \text{H}_2\text{O}_2 \).
Appendix A: Process details for the templated electrodeposition of metals

Template parameters Whatman Anodisc 13 AAO templates: Whatman Anodisc 13 AAO templates with pore diameters of 0.2 µm (template diameter = 13 mm, thicknesses = 60 µm) are very commonly used for making rods and wires. The resulting nanomaterials have a relatively wide range of diameters (180 – 250 nm). An additional downside to using these templates is that their pores are not monocolumnar throughout the thickness of the membrane. A highly branched region exists at one of the two membrane faces, and in order to form rods/wires with uniform lengths this branched region must be filled with metals before beginning deposition of the desired materials. Typically, Ag is deposited into this branched pore region, as the working electrode thermally evaporated onto one template face is also made of Ag and the two Ag regions can be etched simultaneously in HNO₃. Despite these complications, Whatman Anodisc templates have the advantage of being highly cost-effective.

Synkera Technologies, Inc. freestanding AAO templates: Synkera technologies, Inc. fabricates AAO membranes with pore diameters 10 – 200 nm. These templates have more uniform pore diameters (for example, 100 ±10 nm) and do not have the branching region found at one face of the Whatman templates. Naturally, these templates are significantly more expensive.

Of course, a breadth of research exists detailing the fabrication of these templates
from high purity aluminum (Masuda and Fukuda, 1995; Li et al., 1998; Lee et al., 2006; Schwirn et al., 2008). Commercially available templates are used here for convenience.

**Preparing templates for deposition:** Prior to electrodeposition a working electrode must be applied to one side of the template. Typically, this was performed via thermal evaporation of 99.999% Ag onto templates oriented at a slight angle with respect to the direction of the evaporating metal. Additionally, membranes with sub-100 nm pore diameters must be wetted prior to deposition. Often, aqueous electrolytes do a poor job of wetting pores at this dimension. Poor initial wetting results in significantly uneven pore deposition and a general decrease in the fraction of filled pores. Consequently, I employ N,N-dimethyl formamide as a pre-treatment, soaking templates for 5 minutes prior to initial deposition. It is also useful to note that the membrane-working electrode adhesion is not insensitive to time, nor environment. Ag-coated templates stored at standard temperature and pressure experienced some delamination of the Ag layer, presumably due to oxidation at the Ag-template interface. The post-backing lifetime of these templates can be increased by storing them under vacuum, or by storing them in an inert gas. Regardless, templates backed with Ag should be used within 10 days of original backing to ensure proper Ag-template adhesion and minimize the risk of depositing amorphous material into the space resulting from electrode peel-off.

**Depositing multisegmented Ni/Cu rods:** In this section goes the work on electrodeposition of Au-Ni-Au rods, Fe rods, and Ni-Cu multilayered wires. Fe-Cu mul-
tilayered nanowires were deposited from an electrolyte solution containing 41.5 g FeSO$_4$·7H$_2$O, 0.5 g CuSO$_4$·5H$_2$O, 14.87 g (NH$_4$)$_2$SO$_4$, 6.18 g boric acid, and 9 g ascorbic acid for a 250 ml volume. This volume was created using N$_2$-bubbled DI water. The solution was rotated using a magnetic stir bar while N$_2$ was continually bubbled into the solution so as to prevent the rapidly occurring oxidation of FeSO$_4$ ions. Importantly, this solution was always backfilled with N$_2$ to minimize Fe oxidation. Additionally, this solution was always used within three days of making the solution, as the material grown the solution is significantly oxidized if older solution is used.

**Microscope stage setup for magnetic rotational manipulation and tracking:**

Imaging of the magnetic manipulation of our Janus nanorods was performed using a Nikon Diophot inverted microscope, with imaging taking place in transmission mode. Particles were loaded into prefabricated wells created by adhering 24 x 50 mm (#1.5) cover glass to one side of a polydimethylsiloxane (PDMS) chamber. The sample material sits in the hollow of the PDMS chamber. A glass cover slide is sealed to the top of the PDMS chamber using vacuum grease to prevent excess evaporation or induced turbulence from the surrounding environment. Magnetic fields are supplied by a permanent 25 x 11 x 5 mm NeFeB permanent magnet (Grade N42, K&J Magnetics, Jamison, Pennsylvania). This magnet was located above the sample and placed approximately 4 cm away from the sample center. This off axis placement ensures that the rotating magnet does not interfere with the path of the transmitted light. The magnet is attached to a Barber-Colman inline gearmotor (Rockford, Illinois), and this motor was controlled by a Pasco Scientific SF-9584A low
voltage AC/DC power supply (Pasco Scientific, Roseville, California). Video data was collected at either 60 or 120 frames per second using a Pulnix PTM-6710CL camera. In-house custom designed video spot tracking software (Video Spot Tracker V06.04, freely distributed online at http://cismm.cs.unc.edu/downloads/?dl_cat = 3 (Taylor, 2009)).
<table>
<thead>
<tr>
<th>Material</th>
<th>Electrolyte solutions</th>
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| Ni       | 1.10 M NiSO$_4$·6 H$_2$O  
0.20 M NiCl$_2$·6 H$_2$O  
0.75 M H$_3$BO$_3$ |
| Ni / Cu  | 0.50 M NiSO$_4$·6 H$_2$O  
0.01 M CuSO$_4$·5 H$_2$O  
0.75 M H$_3$BO$_3$ |
| Fe       | 0.50 M FeSO$_4$·7 H$_2$O  
0.75 M H$_3$BO$_3$  
0.01 M C$_6$H$_8$O$_6$ |
| Fe / Cu  | 0.50 M FeSO$_4$·7 H$_2$O  
0.01 M CuSO$_4$·5 H$_2$O  
0.75 M H$_3$BO$_3$ |
| Ag       | 1025 RTU @ 4.5 troy/gallon Technic Inc. |
| Au       | Orotemp 24 RTU Rack Technic Inc. |
Bibliography


