Development of Injectable, Stimuli-Responsive Biomaterials as Active Scaffolds for Applications in Advanced Drug Delivery and Osteochondral Tissue Regeneration

A Thesis Presented

By

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Abstract

Osteoarthritis (OA) is a degenerative joint disease that occurs when the cartilage matrix begins to breakdown. Every year, over 3.1 million surgeries are performed in an effort to treat damaged cartilage tissue. Current treatment options such as osteochondral tissue grafting, micro-fracturing, and total knee replacements (TKRs) are effective in alleviating symptoms associated with OA, but often fail to promote the regeneration of normal cartilage. Therefore, due to the limitations of the current treatment options available, it has become necessary to develop better medical solutions to restore or regenerate cartilage tissue previously damaged by OA.

Stimuli-responsive hydrogels, capable of exhibiting dramatic changes in swelling behavior, network structure, permeability and mechanical strength in response to changes in their local environment, have emerged as potential candidates as active scaffolds for several tissue engineering applications. Magneto-responsive biomaterials have become a subject of interest in the field of tissue engineering as their physical and structural properties could be manipulated spatiotemporally by varying the magnetic field strength, making them useful for applications in advanced drug delivery and osteochondral tissue regeneration. Thus, the goal of this thesis was to investigate the feasibility of developing an injectable, magneto-responsive hydrogel scaffold capable of delivering viable stem cell populations to a cartilage defect, and to spatiotemporally control the regeneration of the cartilage tissue in vivo.

A magneto-responsive biomaterial was made by adding functional paramagnetic iron (III) oxide Fe₃O₄ nanoparticles into a thermosensitive pNiPAAm-based hydrogel with degradable PAMAM-based crosslinking macromers. From our tangential force
measurements, we were able to determine that, under a low magnetic field of 0.3 tesla, physically-relevant engineering stresses of 52.5 Pa and 29.6 Pa were generated for hydrogels with 625 μg/mL of 50 or 500 nm nanoparticles (NPs), respectively. Furthermore, primary Mesenchymal Stem Cells (MSCs) were encapsulated within the nanocomposite hydrogel up to seven days, showing that the inclusion of the nanoparticles had no significant impact on MSC viability. These results show that these magnetic hydrogels could be used as injectable scaffolds that permit real-time spatiotemporal control of deformation of the hydrogel, leading to the physical stimulation of encapsulated cells.

Next, to increase macro-porosity within the scaffold, degradable gelatin micro-particles were added to the magnetic hydrogel formulation. Addition of the micro-particles had no adverse effect on cellularity, gelation kinetics or hydrogel formation. However, the micro-particles did have a diminishing effect on the magnetic saturation of our magneto-responsive hydrogels. Furthermore, the feasibility of using magnetic field to accelerate the release of a therapeutic agent from the GMP-composite magnetic hydrogels was investigated.

Finally, the effects of short term magnetic stimulation on stem cell differentiation were also explored. Results showed that magnetic stimulation increases cellularity and hydrogel calcification increased with increases in NP loading but decreased with increases in magnetic stimulation. Furthermore, Alkaline Phosphatase (ALP) expression increased with NP loading, but decreased with magnetic stimulation. It is our hope is that the results presented in this thesis would encourage other scientists to explore using novel stimuli-responsive biomaterials to restore severely damaged tissues.
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Chapter 1: Introduction
1.1 Global Impact of Osteoarthritis

In 1743, world-renowned physician and anatomist, Dr. William Hunter, presented an article to the British Royal Society where he stated that, "Since Hippocrates and until now, it is universally accepted that the ulcerated cartilage … once destroyed, cannot be repaired."\(^5\) Regrettably, this bold statement made over two centuries ago remains true even in our world today, and despite the tremendous advances made in modern day healthcare, the restoration of damaged cartilage tissue still remains an ongoing challenge.

Osteoarthritis (OA) is a degenerative joint disease that occurs when the cartilage matrix begins to break down (Fig. 1).\(^6\) This deterioration of the articular cartilage matrix progressively leads to the loss of function in a load-bearing joint,\(^7\)\(^-\)\(^8\) causing chronic disability in the lower extremities.\(^9\) Cartilage damage caused by OA is primarily initiated through injury, obesity, genetic defects or aging.\(^10\) Archeological excavations of prehistoric Neanderthal fossils (dated to be over 40,000 years old) have shown evidence of cartilage degradation in the elbow joint, suggesting that OA has existed in our world for centuries, affecting even the most primitive of human civilizations.\(^11\)

Today, the World Health Organization (WHO) currently estimates that over 40% of the global population aged 70 or above suffer from OA, making OA the 11\(^{th}\) most frequent cause of disability in the world.\(^12\)\(^-\)\(^13\) In the United States of America, OA affects over 27 million people (Fig. 2) and by the year 2030, it is projected that this number will
rapidly increase to 67 million if new treatment methods to restore damaged cartilage are not readily available.\textsuperscript{14}

\textbf{Figure 2}: Demographic created by the Center of Disease Control and Prevention showing a visual presentation of the prevalence of OA across the United States (2014).

On a global scale, the financial impact of OA has had a crippling effect on the world’s economy. Recent reports have shown that the economic cost associated with OA-related medical expenses have significantly increased around the world over the past several years. In France, the overall financial cost of OA-related medical expenditures exceeded €1.6 billion in 2002 causing a major burden on the French health care system.\textsuperscript{15} Moreover, a study conducted in 2003 estimated that the cost of OA medical expenses in Hong Kong was around $3.2-$3.9 billion, which translates to 0.28\% of Hong Kong’s Gross National Product (GNP).\textsuperscript{16} However, the United States leads the world in OA related expenditures, spending an incredible $22.6 billion in 2004.\textsuperscript{17} This financial burden on the global economy has prompted intergovernmental agencies like the United Nations to act by developing educational campaigns to advance the understanding and treatment of musculoskeletal
disorders through prevention, education and research.\textsuperscript{17-18} With the Baby Boomer generation advancing in age, it is inevitable that the cost of OA related treatments will continue to increase unless better treatment options to regenerate/restore damaged cartilage tissue are made available clinically.

1.2 OA Symptoms and Current Treatment Options

Injuries sustained to the articular cartilage are often problematic to heal due to cartilage’s innate avascular and complex tissue structure. OA can affect any articulating joint in the body, but mainly occurs in the knee, hip, lower back and neck.\textsuperscript{19} Although OA symptoms may vary from patient to patient, people suffering from OA generally tend to have the following symptoms (\textit{Fig. 3}):

- Limited range of motion or stiffness
- Clicking or cracking sound when a joint bends
- Joint swelling
- Pain

Some patients suffering from OA manage their pain by using over the counter drugs such as Acetaminophen (active ingredient in Tylenol and Panadol). However, these medications are costly and more importantly, ineffective in repairing damaged cartilage tissue. Other patients use non-steroidal anti-inflammatory drugs (NSAIDs) such as Ibuprofen or Aleve to treat OA. NSAIDs work by inhibiting the production of enzymes (cyclooxygenase (COX)) by the body, thus reducing inflammation and relieving pain in the short-term. However, the total cost of purchasing NSAIDs for OA runs into almost $4 billion every year, and concerns about the adverse side effects of NSAIDs on the gastrointestinal tract have deterred patients away from their use.\textsuperscript{14}
In more severe cases of OA, surgical intervention may be needed. Currently, there are multiple treatment methods that can be used to treat the symptoms of OA. Some of the most common surgical procedures are:20-21

1. **Arthroscopic lavage** (*Fig. 4a*): In this procedure, a fluid is injected into the affected area to remove inflammatory mediators and other debris. However, this procedure only provides symptomatic relief but does not repair/regenerate normal hyaline cartilage.

2. **Micro-fracturing** (*Fig. 4b*): In this procedure, multiple holes are drilled into the subchondral bone to increase blood flow into the affected area. Bone marrow contains mesenchymal stem cells that could potentially differentiate to form articular cartilage. However, this procedure leads to the formation of fibrocartilage, which is mechanically inferior to the normal hyaline articular cartilage, and often shows signs of degradation within one year.

3. **Osteochondral grafting** (*Fig. 4c*): Cartilage tissue is harvested from the patient (autografts) or from another individual/cadaver (allografts), and transplanted into the osteochondral defect. Autographs require the transplantation of cartilage from non-load bearing cartilage joints into the cartilage defect. The disadvantage of using autographs to treat OA is that they often lead to donor site morbidity, and the patient has to undergo multiple surgeries. Allografts, on the other hand, may induce unwanted immune responses in the host.

4. **Total Knee Replacement (TKR)** (*Fig. 4d*): This is the most common form of surgical treatment for OA. In this procedure, the entire knee joint is replaced with an artificial implant. The surgery is expensive (approximately $75,000) and is only performed as a last resort when the cartilage in the knee has been severely damaged by OA.22 However, this
procedure can cause many complications such as permanent nerve damage in the knee joint, and in some extreme cases pulmonary embolisms – a blockage in the lung’s main artery caused by thromboembolism (local clotting or coagulation of blood). Furthermore, in 2003, 33,000 out of 418,000 (~8%) of the TKRs performed that year had to be surgically revised due to implant failure or infection. However, newer TKR designs have had a high success rate and provide a greater range of motion.

Unfortunately, all the aforementioned surgical treatment options do not restore full mechanical functionality or regenerate/restore normal hyaline cartilage in the knee joint. Therefore, there is a need for better treatment methods to be developed to regenerate damaged cartilage tissue.

1.3 Emerging Technologies in OA Treatment

Due to the growing demand for better clinical solutions to treat damaged cartilage, a few biopharmaceutical companies/startups have ventured into developing novel biomedical
products that could be used to treat OA. Here are a few examples of some products that are either available commercially presently or coming to market soon.

1. **MACI (Vericel):** MACI is a FDA approved cell therapy used to repair small cartilage legions if there is still enough of a cartilage wall present at the site of the defect. Autologous cultured chondrocytes are cultured *in vitro* and used to repair cartilage defects of the femoral condyle. This procedure is used in conjunction with debridement, placement of the periosteal flap (a membrane that covers the outer surface of all bones) and rehabilitation. However, the implementation of MACI requires open surgery, and unfortunately, in several cases there was an overgrowth of cartilage cells in the defect which led to the necessity of a secondary surgery to remove the excess cartilage.

2. **Zilretta™ (Flexion):** Inter-articular corticosteroids remain widely used for symptomatic treatment of peripheral joint OA. Flexion, a biotech startup based out of Burlington, M.A., have developed Zilretta™, an inter-articular corticosteroid that has provided a clinically relevant improvement in pain relief of knee OA. Based on their current scientific finding, Flexion has a Fast Track Designation from the FDA for Zilretta™ and is currently undergoing clinical trials. However, inter-articular corticosteroids can only provide symptomatic relief and do not repair/restore damaged cartilage tissue.

3. **NeoCart (Histogenics):** NeoCart is an implant made from the patient’s own cartilage cells harvested from a non-weight bearing joint. The autologous chondrocytes are seeded on a Type I Collagen scaffold, and subsequently incubated in a bioreactor environment designed to simulate the variation in mechanical forces
and low oxygen tension of the knee (Fig. 5).\textsuperscript{3,27-28} NeoCart is currently in Phase III clinical trials, however, this procedure still requires the patient to undergo multiple surgical procedures, and thus does not provide an attractive solution in the long term.

Figure 5: Schematic showing the formation of NeoCart. First the cartilage is harvested, then expanded on a Type I Collagen scaffold before being incubated in a bioreactor that can simulate the mechanical forces experienced in the knee. The finished implant is surgically placed back into the patient’s knee\textsuperscript{3}.

While these emerging technologies have shown some positive results during their clinical trials studies, most of these proposed therapies are still invasive and would require open surgery. With these shortcomings in sight, it has become of interest in the field of tissue engineering to discover inexpensive, minimally-invasive solutions that could help repair and restore ulcerated cartilage tissue back to its original state.
Chapter 2: Literature Review
2.1 Implantable Scaffolds

When a tissue is damaged to the extent that the use of pharmaceutical treatments alone cannot restore the tissue back to its original state, tissue engineering principles can be used to create, restore, or regenerate the damaged tissue and promote the natural healing process. Dr. Robert Langer (M.I.T) defined tissue engineering as an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ. The regeneration of articular cartilage has been a primary target for tissue engineering efforts due to the lack of functional regeneration in the joint. Over the past two decades, expansions in biomaterial technologies, cell sources, and molecular and genetic manipulations have made a positive impact on the development of functional tissue-engineered cartilage substitutes.

The successful regeneration of cartilage is abundantly dependent on the scaffold implemented to guide tissue development. Scaffolds used for cartilage injuries must not only be biocompatible, but also promote good integration with the host tissue. The primary focus in tissue engineering thus far has been in constructing implantable scaffolds with the appropriate mechanical fortitude to withstand the strong forces experienced in the knee. Various techniques such as 3-D bioprinting, electrospinning, and multilayered structures have been used to fabricate implantable scaffolds for cartilage regeneration.

2.1.1 3-D Bioprinted Scaffolds

3-D bioprinted implantable scaffolds could be used to spatially control the cellular and extracellular matrix (ECM) scaffold arrangement to closely mimic the structure of native cartilage tissue. There are multiple types of 3-D bioprinting strategies (Fig. 6):
• Inkjet bioprinting - droplets containing biological materials are forced out of the nozzle onto a mechanical stage

• Microextrusion bioprinting - a pneumatic or mechanical system is used to extrude a continuous stream of the biomaterial into different geometries

• Laser-assisted bioprinting (LAB) - lasers are implemented to focus a substrate onto a surface

The bioprinting strategy utilized to make the scaffold will be highly dependent on factors such as the surface resolution needed and the material used for printing. The advantage of using a 3-D bioprinter to make an implantable scaffold for osteochondral tissue regeneration is that scaffolds can be created to imitate the complex heterogeneous structure of articular cartilage.

Figure 6: The 3 major different strategies used in 3-D bioprinting which are inkjet (a), microextrusion (b) and laser-assisted bioprinting (c).

Furthermore, bioprinting usually uses naturally derived biomaterials, such as collagen, fibrin, and elastin, in cell-printing solutions as they have superior biocompatibility properties. However, many of these naturally derived materials also have inferior structural and mechanical properties compared to synthetic biomaterials such as polyglycolic acid and poly (ε-caprolactone) (PCL), which have also been vastly used in 3D bioprinting. Thus, researchers have sought to find ways to bioprint scaffolds by combining both natural and synthetic materials. For example, Schuurman et al. (2011) successfully made a bioprinted
scaffold construct with tunable mechanical properties using polycaprolactone (PCL) and cell-laden alginate that had a Young’s modulus (a measure of a material’s resistance to being deformed elastically) of about 6 MPa, which is higher than the normal young’s modulus of cartilage (4.1 MPa). Furthermore, in this study, a short term cell viability study was performed where they found that 60% of the cells survived after 3 days following the bioprinting procedure, but no explanation was given to address why cell viability was decreasing over this short period of time.  

However, the disadvantage of 3-D bioprinting is that there are only a limited selection of materials that can undergo the printing process and maintain the desired mechanical and functional properties. Furthermore, to ensure cell viability, the scaffold must promote angiogenesis (the formation on new blood vessels from preexisting ones) so that the cells within the scaffolds can survive long-term, however, this is a quality that many 3-D bioprinted scaffolds lack.

### 2.1.2 Electrospun Scaffolds

In this approach, ultra-fine fibers are produced by electrically charging a suspended polymer solution. The charged solution is directed towards a grounded collector plate and as the solvent evaporates, dry fibers are formed, collected and fabricated into scaffolds for cartilage regeneration (Fig. 7). Electrospun fibers can be made from various materials and
properties like the fiber morphology and diameter can be easily controlled by adjusting the applied electric field, polymer concentration and/or the solution feed rate.

Figure 7: Electrospinning apparatus showing how polymer fibers are formed.

Scaffolds fabricated via electrospinning are reproducible, and can be made from both natural and synthetic biomaterials. In terms of cartilage regeneration, there have been a lot of published reports that advocate for the use of electrospun scaffolds for cartilage repair since their structure can be made to resemble the natural ECM found in articular cartilage. For example, McCullen et al. (2012) were able to electrospin a collagen scaffold that mimicked the zonal tensile properties of articular cartilage and supported chondrocyte viability and proliferation.\(^{32}\) Furthermore, a recent study by Fisher et al. (2013) showed that nanofibrous scaffolds that could be used to repair a damaged knee meniscus were created by electrospinning PCL. The PCL electrospun scaffold also exhibited both macroscopic and microscopic architecture similar to that of articular cartilage.\(^{33}\) However, the limitations with electrospun osteochondral scaffolds is that complete cellular infiltration throughout the entire scaffold remains an ongoing challenge. Furthermore, making scaffolds of multiple layers by mixing electrospun nano- and micro-fibers remains an obstacle that still needs to be overcome.
2.1.3 Multilayered Structured Scaffolds

Several studies have used a multilayered scaffold design approach to create implantable scaffolds. Articular cartilage is a complex, heterogeneous tissue composed of multiple layers. The benefit of using a multilayered scaffold structure is that it can be made to have the similar distinct zonal characteristics of articular cartilage. In a study performed by Nguyen et al. (2011), a complex, three-layered scaffold composed of a poly(ethylene glycol) (PEG) based hydrogel with chondroitin sulfate (CS), matrix metalloproteinase-sensitive peptides (MMP-pep) and hyaluronic acid was created to have similar mechanical and biochemical properties as native cartilage (Fig. 8).\(^{34}\) From their findings, a high cell viability was maintained and they observed an increase in glycosaminoglycan (GAG) production for over 6 weeks. Unfortunately, the problem with multilayered scaffolds for osteochondral tissue regeneration applications is that defects of irregular shapes remain an ongoing challenge. Furthermore, they require also open surgery.

Figure 8: A multilayered scaffold structure schematic taken from a paper published by Nguyen et al. (2011)\(^ {34}\).

Other implantable scaffold fabrication techniques including micro-particle sintering, freeze-drying, and melt processing have also been used to create implantable scaffolds for cartilage regeneration. However, even though implantable scaffolds have similar mechanical properties and ECM architecture to that of native cartilage tissue, these scaffolds still require open surgery. Moreover, several research studies in the literature
have shown that implantable scaffolds have poor cartilage integration with the host tissue leading to a fibrous capsule forming around scaffold. For example, consider work by Asikainen et al., (2015). In this study a poly(desaminotyrosyl-tyrosine-ethyl ester carbonate) (PDTE carbonate) membrane was implanted into a New Zealand white rabbit for 52 weeks, and a fibrous capsule of about 100 µm thick was formed around the implant (Fig. 9) prohibiting the integration of the scaffold with the host tissue. Therefore, it is has become of prominent attention for the scientific community to devise a new method to synthesize an effective yet minimally invasive scaffold and reduce the chance of infection.

Figure 9: Picture shows a 100-micron fibrous capsule formed around a PDTE carbonate membrane.

2.2 Injectable Scaffolds

Recent tissue engineering efforts have sought to use injectable scaffolds in lieu of implantable scaffolds for cartilage tissue defects. This is because, unlike implantable scaffolds, injectable scaffolds are minimally invasive, can conform to complex tissue defects, and promote good integration with the host tissue. Injectable materials capable of delivering viable cell populations and therapeutic agents into a tissue defect are promising candidates as scaffolds for cartilage regeneration. Injectable biomaterials for cartilage repair can be largely categorized into two main material groups: naturally-derived biomaterials and synthetic biomaterials.
2.2.1 Naturally derived biomaterials

Naturally derived biomaterials are materials commonly found in the body or nature. In terms of cartilage regeneration specifically, there has been a tremendous amount of published literature advocating for the use of naturally derived biomaterials as injectable scaffolds. Naturally derived biomaterials such as collagen, fibrin and hyaluronic acid are commonly used in osteochondral tissue reconstruction because these materials are also found in cartilage and bone tissues. For example, collagen is the most abundant protein found in mammalian tissues and is the main component of the ECM of cartilage. However, there are many challenges with using materials such as collagen, fibrin or gelatin as scaffolds for cartilage regeneration. For example, they have been shown to have inferior mechanical properties when compared to synthetic materials.

Other naturally occurring biomaterials such as agarose (a derivative of seaweed), alginate (a derivative of algae), and chitosan (a derivative of chitin) have also been used as injectable scaffolds. Alginate can ionically crosslink with divalent cations (Ca\(^{2+}\), Mg\(^{2+}\), Ba\(^{2+}\)) to form alginate gels. This simple, tunable gelation mechanism allows for optimization of the mechanical and biodegradation properties of injectable alginate-based gels. However, the problem with alginate is that it undergoes a slow and unpredictable dissolution process in vivo thus limiting its use in real world applications.

Chitosan, on the other hand, has also been effectively used as injectable scaffolds for cartilage regeneration as chitosan is cationic in nature and is similar to normal articular cartilage in its ability to develop electrostatic interactions with anionic glycosaminoglycan (GAGs), proteoglycans and other negatively charged molecules. This is important because GAGs play a pivotal role in modulating chondrocyte morphology, differentiation, and
Chitosan can also form reversible gels in response to changes in pH as chitosan solutions exhibit a liquid to gel transition at a pH of 7 when moving from a slightly acidic to neutral environment. However, chitosan has poor solubility properties in water due to its strong intermolecular hydrogen bonding, thus limiting its use in biomedical applications. As a result of the limitations of naturally-derived biomaterials, researchers have sought to develop synthetic materials with better mechanical, degradation and functional properties as injectable scaffolds for cartilage repair.

2.2.2 Synthetic biomaterials

Synthetic biomaterials are materials that can be created in a laboratory and tailored to carry out a specific function or purpose. When synthesizing a material intended for biomedical applications, several factors, including injectability, solidification mechanism, and the potential for toxic chemical crosslinkers that could possibly leak into neighboring tissues, must be taken into consideration to ensure that the biomaterial meets its intended function. Polymers are generally used as synthetic biomaterials because they are easily reproducible and their mechanical and degradation properties can be manipulated by changing polymer chemistry. However, polymers made for any biomedical applications must have molecular weights below the renal molecular weight cutoff size (~48 kDa in their soluble state) to facilitate easy clearance out of the body through the renal system.

There is an extensive catalog of polymeric materials that have been used for various biomedical applications. In terms of cartilage regeneration, polymers such as poly(ethylene glycol) (PEG), poly(N-isopropylacrylamide) (PNiPAAm), poly(vinyl alcohol) (PVA), poly(propylene fumarate) (PPF) etc. have been commonly used as injectable scaffolds.
Moreover, the list is continually growing as researchers are constantly developing novel synthetic biomaterials for tissue engineering applications. However, synthetic biomaterials do have some significant disadvantages as well, as sometimes multiple purification steps are needed to ensure the removal of any unreacted cytotoxic components left after polymerization.

Moreover, upon injection into the body, injectable polymeric scaffolds generally necessitate a means of solidifying their constituent precursors or macromonomers into a 3-D matrix to influence cellular behavior within the scaffold. These gelation/solidification mechanisms directly affect the mechanical properties and biostability of the resultant scaffolds post-gelation. Gelation of injectable synthetic scaffolds can be initiated by either chemical or ionic crosslinking, or as a response to changes in temperature, pH, or light. The gelation mechanism used to solidify the injectable material is dependent on the type of biomaterial/s used and the intended future application of the injectable scaffold.

**2.2.3 Hydrogels**

A hydrogel is a gel network, composed of either naturally-derived or synthetic biomaterials, that contains a high-water content while maintaining its structure. Hydrogels have been used extensively for many tissue engineering applications because they have been proven to encapsulate viable cell populations, have efficient mass transfer properties, and possess physical properties that can be easily manipulated. Due to these reasons, hydrogels have been used to engineer a wide variety of tissues including cartilage, bone, muscle, fat, liver and neurons. A benefit to using hydrogels as injectable scaffolds for cartilage regeneration is that hydrogels can provide a synthetic ECM structure which helps encapsulated chondrocytes maintain cellular phenotype and morphology. Furthermore, it
has been proven that hydrogels can promote angiogenesis, which is essential for the formation of new tissue.\textsuperscript{48-49}"

Hydrogels composed of biomaterials that can respond to an external stimulus are called stimuli-responsive hydrogels (note: some papers call stimuli-responsive hydrogels ‘smart’, ‘intelligent’ or ‘environment-sensitive’ hydrogels). Stimuli-responsive hydrogels can exhibit dramatic changes in swelling behavior, network structure, permeability and mechanical strength when subjected to even the slightest of changes in their local environment.\textsuperscript{50} Environmentally sensitive hydrogels have been used extensively for various drug delivery applications and can be found in review papers written by Peppas \textit{et al.} (2000),\textsuperscript{50} Bawa \textit{et al.} (2009),\textsuperscript{51} and Langer \textit{et al.} (2012).\textsuperscript{52} However, the potential benefits of using injectable stimuli-responsive hydrogels for tissue regeneration applications are just beginning to be investigated.

\textbf{2.3 Stimuli-Responsive Biomaterials}

Stimuli-responsive biomaterials capable of responding to physical and/or chemical changes in their local environment have emerged as promising candidates for theragnostic applications in drug delivery,\textsuperscript{53} tissue engineering,\textsuperscript{54} and cancer thermotherapy.\textsuperscript{55} While some biomaterials are naturally susceptible to changes in pH and/or temperature (due to their innate thermodynamic properties),\textsuperscript{56} other biomaterials can be made to respond to an external stimulus such as light,\textsuperscript{57} sound,\textsuperscript{58} or magnetic fields.\textsuperscript{53, 59} This can be achieved by incorporating specialized nanoparticles into the biomaterial network to initiate dramatic changes in shape/swelling, network structure and mechanical integrity.\textsuperscript{50, 60} In this section, a brief discussion on how stimuli-responsive biomaterials (particularly thermo-responsive and magneto-responsive biomaterials) have been implemented in tissue engineering.
2.3.1 Thermo-responsive Biomaterials

Temperature sensitive polymers are probably the most commonly studied class of stimuli responsive polymer systems for tissue regeneration purposes. Thermogelling macromers (TGM) that undergo a coil-to-globule phase transition at a Lower Critical Solution Temperature (LCST) close to body temperature have been widely used as injectable scaffolds because of their near-instantaneous gelation that occurs due to a sudden elevation in local temperature. This phase transition happens because it is much more thermodynamically favorable for the solution to exist in two phases as the hydrophobic interactions among the hydrophobic segments dominate over the hydrogen bonding between the hydrophilic polymer segments and the surrounding water molecules. Specifically, if we consider the Gibbs equation $\Delta G = \Delta H - T\Delta S$, the driving force for the phase transition is the entropy of the water, so that, when the polymer is not in solution, the water is less ordered and has a higher entropy.\(^6\) This is also called the hydrophobic effect.

One of the most commonly used thermoresponsive polymers is poly($N$-isopropylacrylamide) (PNiPAAm). PNiPAAm has an LCST close to body temperature (~32°C), and can solidify \textit{in situ} without the addition of cytotoxic crosslinkers, initiators, or
catalyst molecules (Fig. 10). Other examples of thermoresponsive polymers include poly($N, N'$-diethyl acrylamide) (PDEAAM) which has an LCST in the range of 25-35°C. However, the LCST is mainly dependent on the tacticity of the polymer. Poly(2-carboxyisopropylacrylamide) (PCIPAAm) is another temperature responsive polymer that has similar behavioral properties to PNiPAAm.⁵⁸

Due to PNiPAAm’s thermal transition properties, PNiPAAm-based hydrogels have been successfully used as injectable scaffolds because they are able to deliver viable cell populations and can undergo a thermal gelation without having toxic chemical crosslinkers in the injectable hydrogel formulation.⁶² However, the challenges that hinder the application of NiPAAm hydrogels from being used as injectable scaffolds are that NiPAAm-based scaffolds have a tendency to undergo syneresis post formation and furthermore, that NiPAAm is not biodegradable. Ekenseair et al. (2012) addressed the issue of syneresis by synthesizing a dual thermally and chemically cross-linked hydrogel composed of a PNiPAAm backbone with a hydrophilic, cytocompatible polyamidoamine (PAMAM) chemical cross-linker.⁶³ In their research, they copolymerized PNiPAAm with glycidyl methacrylate (GMA) to form TGMs with pendant epoxy groups which reacted with the amine groups located on the PAMAM, thus forming degradable crosslinks in the hydrogel network. They found that by utilizing PAMAMs as degradable crosslinks they could tune hydrogel swelling/syneresis, degradation time scale and degree of crosslinking (Fig. 11).⁶⁴
To address the issue of non-degradability of the PNiPAAm-based hydrogels, many research groups have attempted to enhance the bioresorability of PNiPAAm polymers by copolymerizing with complex with pendant groups such as lactate esters or polyester side groups that modulate the LCST over time via hydrolysis. However, these monomers produced soluble degradation products that could be potentially toxic to the encapsulated cells and raise the local pH micro-environment. Cui et al. (2007) achieved PNiPAAm biodegradation without toxic byproducts through incorporation of dimethyl-γ-butyrolactone acrylate (DBA), a hydrolysable pendant lactone ring. Hydrolysis of the ester group in the ring structure resulted in the formation of hydroxyl and carboxyl groups which increased the hydrophilicity of the polymer, thus ultimately increasing the LCST of the polymer post formation.

To this point, recently Vo et al. (2014) first successfully synthesized a PNiPAAm-based hydrogel with DBA incorporated in the polymer network and demonstrated the hydrogel’s capacity to support MSC viability for up to 7 days without affecting the hydrogel crosslinking. In a subsequent study, Vo et al. (2015) used the same injectable, acellular hydrogel formulation to fill an 8mm critical size rat cranial defect. After 12 weeks, the hydrogels were taken out and analyzed for bone formation, hydrogel mineralization and

Figure 11: Hydrogel formation and syneresis with 10 wt.% TGM and varying PAMAM content (0-10 wt.%) with volume swelling ratio relative to 0% PAMAM.
tissue response using microcomputed tomography. Fig. 12, an image from Vo’s 2015 publication in the Journal of Controlled Release, shows the cross-section of a p(NiPAAm-co-DBA-co-GMA-co-AA) hydrogel scaffold verifying bone formation through von Kossa and hematoxylin and eosin (H&E) histological staining over time and over varying polymer compositions, thus proving that PNiPAAm hydrogels are good candidates for osteochondral tissue regeneration.

Figure 12: Picture showing cross-sectional surface of Vo et. al. PNiPAAm-co-DBA hydrogel scaffold that verifies bone formation through von Kossa and hematoxylin and eosin (H&E) histological staining over 12 weeks. They found that their hydrogels showed signs of mineralization across the whole defect and there were minimal inflammatory responses. 15wt.% TGM/DBA (left column), 20 wt.% TGM/DBA (middle column), and 15wt.% TGM hydrogels (right column) at 4 (A–C) and 12 (D–F) weeks.

2.3.2 Magneto-responsive Biomaterials

Magneto-responsive biomaterials that can be manipulated spatiotemporally via an external magnetic field have emerged as promising candidates as active scaffolds for advanced drug delivery and tissue regeneration applications. These specialized biomaterials can be
synthesized by physically and/or chemically incorporating MNPs into the biomaterial structure. However, despite their promising impact on the future of regenerative medicine, magneto-responsive biomaterials still have several limitations that need to be overcome before they can be employed clinically as it is difficult to predict their behavior in vivo. This section will focus on discussing the current fabrication methods used to synthesize magneto-responsive materials, the governing equations that can be used to predict the biomaterial’s behavior in the body, and the potential biomedical applications of magnetic biomaterials as controlled drug delivery systems, tissue engineering scaffolds and soft actuators.

Magnetic nanoparticles (MNPs) are particles (< 100 nm in size) composed of magnetic elements, such as iron (Fe), nickel (Ni), cobalt (Co), or their oxides. Although an extensive amount of research has been conducted to evaluate their unique physical, structural and magnetic properties, the scientific community is still at the threshold of understanding the full potential impact that MNPs can have on the future of medicine. Recent literature (circa 2010) has explored advantages of using MNPs for biomedical applications in targeted drug delivery, hyperthermia, magnetic resonance imaging, cellular targeting and tissue engineering. For example, it has been documented that functionalized MNPs can be bound to cellular surface receptors, and by subjecting these particles to an external magnetic field, mechanical forces can be transmitted to the cytoskeleton to trigger an increase in ion channel activity (Fig. 13). Hughes et al. (2005) demonstrated this as they used MNPs to activate mechanosensitive ion channels on the cellular membrane to activate the biochemical pathways that directly influence osmoregulation.
Figure 13: MNPs can be bound to the cell receptors located on the cell membrane surface. When a magnetic field is applied, the MNPs generate mechanical forces that can be transmitted to the cell membrane to activate mechanosensitive ion channels. However, despite the tremendous influence that MNPs could potentially have on the future of biomedicine, several questions concerning their cytotoxic effects on biological cells/tissues have limited their progress in advanced clinical trials.\textsuperscript{77} MNPs (especially particles < 50 nm in diameter) are able to diffuse across biological membranes and initiate adverse cytotoxic effects in the biological tissue such as inflammation, formation of apoptotic bodies, impaired mitochondrial/DNA function, membrane leakage of lactate dehydrogenase and/or generation of reactive oxygen species.\textsuperscript{78} Due to these limitations, scientists have shifted their focus to develop next-generation, stimuli-responsive biomaterials that still possess unique magnetic properties which can be controlled spatiotemporally without having unfavorable outcomes on cell viability.

‘Magneto-responsive’ biomaterials can be synthesized by either physically or chemically incorporating MNPs into the biomaterial structure, and by varying the magnetic field strength gradient, one can theoretically spatiotemporally control the physical, structural and mechanical properties of these magnetic biomaterials making them useful for a multitude of biomedical applications, such as guiding the regeneration of complex
heterogeneous tissue structures in vivo. Moreover, since the MNPs are bound to the biomaterial structure, concerns about the cytotoxicity and nanoparticle internalization in neighboring cells are significantly reduced. Magneto-responsive biomaterials have already gained a lot interest for their potential applications in on-demand drug delivery,\(^5\), \(^6\), however, more and more scientists are exploring their prospective applications in other areas of regenerative medicine.

2.3.2.1 Basic Magnetic Force/Torque Concepts

Forces transmitted onto a cell membrane via MNPs can be created either by producing shear forces that occur as a result of magnetic drag forces,\(^8\), \(^9\) or magnetic torque/rotation produced by orientating the magnetic field at an angle to the particles’ magnetization vector.\(^7\) Several attempts have been made to model the magnetic force generated on a single particle at a given magnetic field strength. Theoretically, the magnetic force \(\vec{F}_m\) generated on a single particle (idealized as a magnetic sphere of uniform density) can be calculated using equation 1.\(^\)\(^3\)

\[
\vec{F}_m = V_p \Delta \chi \nabla \left( \frac{\vec{B}^2}{2 \mu_0} \right) = \frac{V_p \chi_p}{\mu_0} \begin{bmatrix} B_x \frac{\partial B_x}{\partial x} + B_y \frac{\partial B_y}{\partial y} + B_z \frac{\partial B_z}{\partial z} \\ B_x \frac{\partial B_y}{\partial x} + B_y \frac{\partial B_y}{\partial y} + B_z \frac{\partial B_z}{\partial z} \\ B_x \frac{\partial B_z}{\partial x} + B_y \frac{\partial B_z}{\partial y} + B_z \frac{\partial B_z}{\partial z} \end{bmatrix} \tag{Eq. 1}
\]

where:  
\(\vec{F}_m\) = magnetic force  
\(V_p\) = nanoparticle volume  
\(\chi\) = volumetric magnetic susceptibility (can be obtained from a Superconducting Quantum Interference Device (SQUID) magnetometer)  
\(\vec{B}\) = applied magnetic field
\[ \mu_0 = \text{permeability to vacuum (constant)} = 4\pi \times 10^{-7} \text{TmA}^{-1} \]

By knowing the magnetic field gradient, magnetic susceptibility of the magnetic biomaterial and NP volume and number of the MNPs in the biomaterial, calculations can be made to theoretically predict the magnetic force that can be generated by the biomaterial.

It is well known that mechano-transduction plays a crucial role in the development of vascular and osteochondral tissue,\textsuperscript{84-86} thus understanding the relationship presented in the above is vital to provide the appropriate frequency of mechanical stimulation needed to promote the regeneration of a damaged tissue. For example, a paper written by Henstock et al. (2014) showed that they were able to improve mineralization in chicken fetal femurs by microinjecting MNPs into the bone tissue and directly delivering 4 picoNewtons (pN) of force per nanoparticle at a low magnetic field strength of 25 millitesla (mT).\textsuperscript{87}

On the other hand, magnetic torque is generated by applying an external magnetic field that is oriented at an angle to the particle's magnetization vector causing a "twisting" effect on the particle (Fig. 14).\textsuperscript{75} This phenomenon can be expressed by Equation 2:\textsuperscript{55}

\[ \vec{T}_m = \frac{\partial U_m}{\partial \theta} = \mu_0 V_p \vec{M} \times \vec{H} \]  

(Eq. 2).

where: \( \vec{T}_m \) = magnetic torque

\( U_m \) = internal energy state of the nanoparticle

\( \theta \) = angle at which magnetic field is being applied

\( \vec{M} \) = magnetization of the nanoparticle

\( \vec{H} \) = magnetic field strength
Note: This equation is only valid for a nanoparticle surrounded in a non-magnetic medium such as air or water. Furthermore, for paramagnetic nanoparticles, there is no permanent magnetic dipole within the material and thus Equation 2 equals zero. A detailed explanation about the derivation of the above equation and other potential applications of magnetic torque in the biomedical field can be found in a review paper written by Erb et al. (2016). Wang et al. (1998) used magnetic torque to actuate MNP-tagged cells. They found that the rotational torque on the MNPs could apply mechanical forces to the cell membrane to activate mechanosensitive cellular responses. However, an overlying challenge hindering the implementation of magneto-responsive biomaterials for regenerative medicine applications is that it is quite difficult to predict the force distribution within the biomaterial even with the use of the mathematical equations presented above. This complication makes it a strenuous task to predict how these materials would respond to a magnetic field in vivo. As one can imagine, depending on how the scaffold was synthesized, the MNPs can be randomly distributed within the biomaterial, and in the presence of a magnetic field, the magnetic force generated in an area with a high concentration of MNPs would be much higher than an area with fewer MNPs. Furthermore, even if the MNPs were hypothetically uniformly distributed within a biomaterial, it would be a challenge to accurately predict the magnetic force/torque generated on a single particle attached to a substrate as the physical/chemical bonds would limit the particles ability to freely move/rotate in the presence of a magnetic field. However, if the biomaterial was elastic in nature and had a known modulus of elasticity (Young’s modulus), the amount of magnetic force needed to deform a scaffold a certain percentage can be estimated using Hooke’s law (Equation 3).

By inserting Equation 3 into Equation 1, approximations of the magnetic field strength needed to elicit a known deformation/engineering strain within the magneto-responsive biomaterial can be calculated. Sapir-Lekhovitser et al. (2016) used the relationship above to estimate that they could generate a deformation of ~10 nm within their magnetic alginate-based scaffold at a low magnetic field strength of 15 Oersted (Oe). They hypothesize that this amount of strain within the scaffold would transmit a localized force of 1 pN on an encapsulated human umbilical vein endothelial cell, which would be enough force to initiate mechano-transduction pathways within the cell. Previous reports have shown that the threshold for cellular mechano-transduction is only 0.2 pN. However, more work is needed to develop more accurate theoretical models that would help us predict a magneto-responsive biomaterial behavior in vivo.

2.3.2.2 Synthesis of Magneto-Responsive Biomaterials

Several strategies have been used to develop magneto-responsive scaffolds. These scaffolds usually consist of biomaterials with MNPs incorporated into the scaffold. This incorporation of MNPs into the scaffold can be achieved by either blending the MNPs with the biomaterial, in situ precipitation of MNPs in a swollen hydrogel environment, or forming covalent bonds between MNPs and a biomaterial scaffold. Moreover, cells can...
either be mixed in with the biomaterial pre-solidification, or seeded onto the scaffold surface post-solidification.

I. Blending: In the blending method, MNPs are physically mixed into the scaffold mixture pre-formation. In a paper published by Sapir et al. (2012), MNPs were suspended in alginate solution to form a magneto-responsive hydrogel. They found that nanoparticles ranging between 5-20 nm tend to aggregate within the scaffold due to strong surface forces and a high surface area.\(^{91}\) Furthermore, Tong and colleagues (2010) could fabricate magneto-responsive hydrogels by blending MNPs with an \(N\)-isopropylacrylamide (NiPAAm) solution. The formed magnetic hydrogels were then separated by an external magnetic field from the NiPAAm solution.\(^{92}\) Moreover, Fuhrer et al. (2013) fabricated magnetic scaffolds by sonicating functionalized nanomagnets in a polymer solution composed of 2-hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA) and styrene maleic anhydride (SMA) (Figure 14).\(^{93}\) However, the main problem when using the blending technique is that it is a challenge to uniformly distribute the nanoparticles within the scaffold. Furthermore, it is possible for the MNPs to diffuse out of the scaffold if immersed in a liquid medium.\(^{54}\)
Figure 14: Magnetic scaffolds fabricated by blending nanomagnets with a polymer solution of HEMA, EGDMA and SMA. a) Carbon protected metal nanomagnets (transmission electron microscopy image) were covalently linked into a hydrogel (b, cryo-section) with high porosity (pore size ~ 10 µm) to facilitate cell attachment. c) Magnetic, soft, cell culture scaffold. The dog-bone shape minimizes the required magnetic force for soft deformation. d) Good adherence of human mesenchymal stem cells seeded on hydrogel surfaces (Calcein-AM staining).93

2. **In Situ Precipitation:** For this technique, first hydrogels are formed by either a physical or chemical crosslinking mechanism, and then swollen in a solution containing Fe$^{2+}$ and Fe$^{3+}$ ions that can be made into MNPs by the following reaction:

$$\text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH}^- \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O}$$

The main advantages of the in-situ preparation technique are that it is relatively inexpensive, and a large amount of MNPs can be uniformly distributed into the scaffold network. Hu *et al.* (2007) were able to make magnetic ferroscaffolds by an *in situ* precipitation process in which iron oxide nanoparticles were co-precipitated and deposited within a gelatin hydrogel.94 They also found that by increasing the amount of iron oxide nanoparticles inside the ferroscaffold, the magnetic properties of the scaffold were enhanced. Hernandez *et al.* (2009) were also able to fabricate a semi-
interpenetrating alginate and poly(N-isopropylacrylamide) (pNiPAAm) hydrogel with iron (III) oxide nanoparticles incorporated in the network by in situ preparation. Furthermore, Wong and coworkers (2008) synthesized magnetic chitosan hydrogels by chelating the ferric and ferrous ions with the amino groups on chitosan (CS) to form a CS-Fe (II, III) precursor. In the presence of hydroxide ions, the magnetite crystals that were formed were dispersed within the chitosan hydrogel. However, in situ preparation of magneto-responsive scaffolds is only possible if the scaffold can maintain its network structure and not be damaged by the strong alkali environment needed to form the MNPs. Furthermore, the strong alkali conditions that are needed to make the scaffold may not be conducive for cell encapsulation applications.

3. **Covalent Bonding:** In this technique, covalent bonds are formed between functionalized MNPs and the biomaterial scaffold. In a paper written by Dr. Patrick Ilg (2013), he proposed the idea of incorporating functionalized cobalt ferrite (CoFe$_2$O$_4$) nanoparticles into a hydrogel composed of different polymers, such as poly(ethylene glycol) (PEG), poly(acrylamide) (PAAm) and polysaccharides, depending on the functional groups present on the nanoparticle surface. In our research lab, we are able to synthesize magneto-responsive nanocomposite hydrogels by incorporating amine-functionalized iron (III) oxide nanoparticles into pNiPAAm-based thermogelling macromers with pendant epoxide rings developed by Ekenseair *et al.* (2012). The key advantage of this technique is that the covalent bonds between the nanoparticles and the scaffold prevent the nanoparticles from leaching out. However, this method is generally less cost-effective than the other methods presented.
4. **Dip-coating:** Recently, Bock *et al.* (2010) presented an alternative method of fabricating magnetic biomimetic scaffolds for bone tissue engineering by dip-coating their scaffold in a ferrofluid (Figure 15). To describe the process briefly, first they created a composite scaffold from hydroxyapatite (HA) and collagen in a 70:30 wt.% ratio. Both of these materials have been regularly used as bone graft substitutes because they have excellent biocompatibility properties and are also naturally found in the body. The formed HA-collagen scaffolds were immersed in a ferrofluid (to allow the fluid to flow and occupy the pores in the scaffold), freeze dried, and washed repeatedly with deionized water. They found that they could significantly improve the magnetic properties of their scaffolds by simply dipping the scaffold in the ferrofluid multiple times. However, more biocompatibility studies need to be conducted to verify that these types of scaffolds are not cytotoxic to cells. Overall, the procedure used to form a magneto-responsive biomaterial is dependent on the ultimate future application of the biomaterial.

![Figure 15: A schematic illustration of how to use dip-coating to magnetize a scaffold before magnetization (scale bar 2 mm). (C) Photograph showing the attraction of the porous scaffold.](image)
biomimetic HA/COLL composite scaffold to a standard magnet after magnetization (scale bar 4 mm). Magneto-responsive biomaterials show high potential as versatile materials for a multitude of biomedical applications. These specialized scaffolds can have an immense advantageous impact in the future of regenerative medicine. However, there are still multiple issues that need to be resolved before magnetic scaffolds can be used clinically. First, more in vivo studies must be done to address cytotoxicity concerns and determine the long-term fate of the MNPs in the body. Secondly, in the presence of an alternating magnetic field, the MNPs may begin to overheat over time causing cell death in the surrounding tissues. Most importantly, more theoretical calculations need to be performed to be able to accurately predict the scaffold behavior in vivo under the influence of a strong external magnetic field.

2.4 Biomedical Applications of Magneto-responsive Biomaterials

2.4.1 Osteochondral Tissue Regeneration

Figure 16: Scheme showing mesenchymal stem cells differentiate into different types taken from discoverymedicine.com.
Mesenchymal Stem Cells (MSCs) are adult stem cells that are traditionally found in the bone marrow or adipose tissue. These specialized cells are valuable because they can differentiate into various cell types such as adipocytes, cartilage, bone, tendon, muscle and skin cells.\textsuperscript{101} MSCs have been comprehensively researched in the field of tissue engineering/regenerative medicine because autologous MSCs will not elicit a severe immune response from the host tissue. A schematic of the different pathways by which MSCs differentiate into other cells is shown in Fig. 16.

Osteogenic differentiation of stem cells can be induced by using either biochemical cues such as growth factors or biophysical cues such as mechanical forces. While growth factors such as bone morphogenic proteins (BMPs), fibroblast growth factors (FGF), insulin-like growth factor (IGF) and transforming growth factor beta (TGF-β), have been used to regulate the development and homeostasis of articular cartilage, one major drawback to using growth factors is the potential for ectopic tissue formation.\textsuperscript{102} Furthermore, growth factors tend to have relatively short half-lives and need to be reloaded constantly to maintain cellular phenotype.

On the other hand, it has been well documented that external forces and matrix mechanics play a crucial role in the regulation of stem cell fate.\textsuperscript{103} Furthermore, it is well known that continuous mechanical stimulation of MSCs, after differentiation to osteoblasts/chondrocytes, is needed to improve their efficacy in joint repair. Research has shown that, by mechanically stimulating the receptors on a stem cell surface, various mechano-transduction pathways such as Ras/MAPK, PI3K/Akt, RhoA/ROCK, and TGF-β pathways are activated causing the stem cells to commit to a chondrogenic lineage.\textsuperscript{104} Different strategies can be used to apply mechanical forces on cells, \textit{in vivo} and \textit{in vitro}. 
Literature has shown that factors such as substrate stiffness, surface nanotopography, and extracellular forces, can all direct stem cell fate *in vitro* even with the absence of growth factors.\(^\text{85}\)

Several techniques have been used to apply external forces on MSCs to initiate osteogenic differentiation. Schätti *et al.* (2011) proposed a technique to apply compressive and shear forces on MSCs by using a ceramic rotating hip ball to cause MSCs to differentiate into chondrocytes (Fig. 17).\(^\text{105}\) Their results showed a significant increase in GAG production over a 3-week period when samples experienced both compressive and shear forces. Other strategies used to exert mechanical forces on cells include dynamic stamp, centrifugal forces,\(^\text{106}\) and the use of hydrostatic pressure to apply cyclic compressive forces on stem cells.\(^\text{107}\) However, these procedures are only practical in an *in vitro* setting. Thus, one of the challenges in the field of stem cell engineering is to develop innovative systems that can guide the regeneration of a cartilage defect by spatiotemporally manipulating the shear and compressive stresses that stem cells experience *in vivo*.

![Figure 17: Left: Cross sectional schematic of the scaffold with its holder. The ceramic hip ball (A) is pressed against the cell seeded scaffold (B). The scaffold (B) is held in place within the main holder (C) by means of a circular PEEK ring (D).](image-url)
One possible alternative to address this problem is to develop magnetic biomaterials capable of generating localized forces with the biomaterial scaffold when subjected to an external magnetic field. Some scientists have already foreseen the potential benefits of using stimuli-responsive biomaterials for tissue regeneration application, and there have been a few published reports advocating for the use of magnetic hydrogels for osteochondral tissue regeneration applications.

- **Bone Regeneration:** Skeletal bone defects caused by excessive force trauma, tumor resection, or disease (e.g. osteoporosis) can be very challenging to repair.\(^{108}\) Bone is a highly complex tissue that needs continual mechanical stimulation to promote and maintain a functional structure.\(^{104, 109}\) Scaffolds play a crucial role in bone regeneration, as they not only provide an environment for cell adhesion but also guide cell proliferation and differentiation.\(^{110}\) Magnetic biomaterials capable of delivering direct mechanical stimulation to individual cells without the need for expensive biomolecules could potentially be used as scaffolds for bone regeneration applications. Meng and colleagues (2013) have shown that by incorporating superparamagnetic nanoparticles in an electrospun hydroxyapatite (HA)/poly(lactic) acid (PLA) scaffold, they were able to support cell attachment and proliferation while under the influence of a weak magnetic field (5-25 millitesla).\(^{109}\) They also found that under an external magnetic field, their scaffold induced earlier and higher amounts of osteocalcin positive cells, which can lead to faster bone formation in a bone defect. Furthermore, Panseri *et al.* (2012) created magnetic scaffolds by impregnating a composite HA-collagen scaffold with a ferrofluid solution.\(^{111}\) From their results, they could show that cells and collagen fibers
aligned parallel to the magnetic field lines under a strong magnetic field of 8 tesla both \textit{in vitro} and \textit{in vivo}. Moreover, Xu and Gu (2014) wrote a great review article advocating for the need for paramagnetic scaffolds in bone regeneration and citing multiple examples from literature on how other scientists have developed magnetic scaffolds to repair bone defects.\textsuperscript{110} However, more \textit{in vivo} studies need to be conducted to evaluate the biocompatibility of these magneto-responsive biomaterials. Also, many of the \textit{in vivo} studies are conducted in small animal orthopedic models, which have inherent differences in bone composition, density and quality when compared to humans. Thus, the results from these animal studies cannot be directly extrapolated to human conditions and investigations in larger animal models are needed.\textsuperscript{112}

- **Cartilage Regeneration:** The restoration of articular cartilage has been a primary target in the field of tissue engineering due to the lack of functional regeneration within the joint. There have been a few published reports supporting the use of magneto-responsive hydrogels for cartilage regeneration. In a study conducted by Fuhrer \textit{et al.} (2013), human stem cells were seeded onto an implantable magnetic hydrogel scaffold. The scaffold was made by suspending nanomagnets into a solution containing ethylene glycol dimethacrylate (EGDMA) and 2-hydroxyethyl-methacrylate (HEMA) and subsequent copolymerization. The scaffolds were then subjected to a controlled magnetic field of 0.8 tesla. They found that the mechanical stimulation resulting from the deformation of the magnetic scaffold significantly increased GAG expression and caused the stem cells to commit to a chondrogenic lineage.\textsuperscript{93}
Furthermore, Sapir-Lekhovitser et al. (2016) developed an implantable magneto-responsive alginate-based scaffold that could undergo reversible shape deformation when exposed to a time-varying magnetic field generated by a Helmholtz coil. They estimated that the mechanical force that could be imposed on cells seeded in the scaffold would be on the order of 1 pN, which correlates to the reported threshold to activate mechano-transduction pathways on a cellular level. However, more work is needed to understand the amount of force that can be generated given the concentration of nanoparticles incorporated into the scaffold. Thus, an accurate model of the force distribution generated by a magnetic field within a scaffold is needed.

Magnetic biomaterials have also been used in cardiovascular and neuronal tissue engineering applications as well. However, even though magneto-responsive biomaterials show high potential as versatile materials for a multitude of biomedical applications that can have an immense advantageous impact on osteochondral tissue regeneration, there are still multiple barriers that need to be resolved before magnetic scaffolds can be used clinically. MNPs cytotoxicity and the long-term fate of the MNPs in vivo post-implantation are all essential questions that need to be addressed before these materials are used clinically. Furthermore, more theoretical models/calculations need to be performed to be able to accurately predict the scaffold behavior in vivo under the influence of a strong external magnetic field.

2.4.2 Drug Delivery
For decades, scientists have sought to develop better controlled drug delivery systems to deliver therapeutic agents periodically at a specific target. Controlled drug delivery systems
capable of releasing drugs in response to fluctuating metabolic requirements or in the presence of certain biomolecules would be highly effective in treating clinical disorders, such as diabetes and rhythmic heart disorders.\textsuperscript{53} To address this problem, many researchers have shifted their focus to developing stimuli-responsive polymeric drug carriers that can be implanted and release drugs on-demand via internal/external signaling. Magnetic biomaterials might potentially be the ideal candidates to solve this problem. Kost \textit{et al.} (1985) published one of the first articles advocating for the use of a magnetic polymeric system to deliver a drug with reproducible regulation.\textsuperscript{114-115} In their work, they embedded small magnetic spheres into ethylene-vinyl acetate (Evac) copolymer and, by using a low magnetic field, released bovine serum albumin (BSA) protein over time. A few years later, Kost \textit{et al.} (1987) demonstrated that they could use this same magneto-responsive biomaterial system for controlled release of insulin to reduce blood glucose levels in diabetic rats.\textsuperscript{54, 116} More recently, Dionigi \textit{et al.} (2014) developed a magnetic, thermo-sensitive nanocomposite hydrogel (made by absorbing MNPs onto the shrunken state of pNiPAAm) for the controlled release of vascular endothelial growth factor (VEGF) under a moderate magnetic field of 0.48T.\textsuperscript{117} Furthermore, Zhao and coworkers (2011) designed active macroporous ferrogels by incorporating Fe\textsubscript{3}O\textsubscript{4} nanoparticles into alginate-based gels.\textsuperscript{79} In their work, they demonstrated that these ferrogels could deliver various drugs and therapeutic agents, both \textit{in vitro} and \textit{in vivo} (Figure 18). However, although magnetic biomaterials can be used to control the pulsatile release of drugs, the efficacy of the drug release would be highly dependent on how close to the target site the magnetic biomaterial can be implanted.\textsuperscript{92} Thus, for patients that have malignant tumors embedded deep within
biological tissue, it may be a challenge to deliver therapeutic agents to the tumor with a magnetic hydrogel.

![Cumulative release profiles of mitoxantrone (A) and plasmid DNA (B) from a microporous ferrogel subject to 2 min of magnetic stimulation every 30 min, or no magnetic stimulation.]

**Figure 18:** Cumulative release profiles of mitoxantrone (A) and plasmid DNA (B) from a microporous ferrogel subject to 2 min of magnetic stimulation every 30 min, or no magnetic stimulation.118

### 2.5 Our Experimental Approach

The primary goal of the research presented in this thesis was to conduct preliminary investigations to determine the feasibility of utilizing stimuli-responsive hydrogels as active scaffolds for applications in advanced drug delivery and osteochondral tissue regeneration. Our hope is that from the experiments conducted, better insights on how MSCs respond to variations in shear and compressive stresses can be determined, thereby leading to the development of novel stimuli-responsive biomaterials capable of guiding the regeneration of a damaged heterogeneous tissue structure *in vivo*.

In our experimental approach (depicted in Fig. 19) an injectable hydrogel scaffold with magnetic properties that could be implemented *in vivo* without surgical intervention was created. Our hypothesis is that the incorporation of biocompatible magnetic nanoparticles into the cross-linked network of a polymeric scaffold will enable non-destructive, real-time stimulation of osteochondral tissue regeneration through mechanical force and
deformation. NiPAAm (a biocompatible, thermo-sensitive macromer) was chosen to be the basis of our hydrogel formulation. NiPAAm has an LCST close to body temperature, allowing it to thermogel instantaneously *in situ* upon injection into the body. A cytocompatible chemical cross-linker, PAMAM was added to the hydrogel formulation to counteract NiPAAm’s natural tendency to undergo syneresis post formation. Functionalized MNPs can then be incorporated into the hydrogel by forming a chemical bond between the nanoparticle and polymer backbone, making the biomaterial responsive to external magnetic fields. MSCs and other therapeutic agents could then be encapsulated within the nanocomposite scaffold by simply adding them to the hydrogel formulation pre-gelation. The spatiotemporal control of nanoparticle loading and utilization of magnetic field gradients will enable heterogeneous cell activity and ultimately the generation of multi-tissue defects from a single minimally-invasive liquid injection.

The primary research aims of this thesis are as follows:

**Research Aim 1:** Synthesis and characterization of an injectable, magnetic hydrogel scaffold. The efficacy of *in situ* hydrogel formation, dimensional stability, mechanical properties, reaction kinetics, tangential force potential, and the effects of polymer and nanoparticle chemistry and loading were investigated.

**Research Aim 2:** Increase macro-porosity within the magneto-responsive biomaterial scaffold by incorporating degradable gelatin micro-particles into the hydrogel structure, and evaluate the efficacy of using our magneto-responsive biomaterials for advanced drug delivery applications.
**Research Aim 3:** Evaluate cellular responses to locally induced shear and compressive stresses generated within the magneto-responsive hydrogel scaffold in response to alterations in magnetic field strength and frequency.

![Diagram](image)

Figure 19: Formation of injectable, magneto-responsive hydrogels composed of thermogelling macromers, iron (III) oxide (Fe₃O₄) NPs and encapsulated mesenchymal stem cells.
Chapter 3: Synthesis and Characterization of Injectable, Magneto-responsive Hydrogel Scaffolds (Research Aim 1)
3.1 Introduction

Recent research efforts have turned to using injectable scaffolds to repair cartilage defects, as they do not require open surgery, can conform to complex tissue defects, and promote good integration with the host tissue. Stimuli-responsive biomaterials, capable of producing changes in mechanical and structural properties as a response to changes in an external stimulus, have shown promise as active scaffolds for applications in advanced drug delivery and tissue engineering. The premise of this first aim was to assess the feasibility of developing an injectable, magnetic hydrogel scaffold that could be used to promote the regeneration of a heterogeneous tissue defect.

As mentioned previously in the literature review (Section 2.3.1), one of the most commonly used injectable biomaterials used in tissue engineering is the thermosensitive polymer, PNiPAAm. PNiPAAm has a lower critical solution temperature (LCST) close to body temperature (~ 32°C) and can solidify in situ without the addition of cytotoxic crosslinkers, initiators, or catalyst molecules. Furthermore, PNiPAAm has also been shown to be capable of delivering viable encapsulated cell populations (specifically chondrocytes) in vitro. It was for these reasons that PNiPAAm was chosen to be the base of our hydrogel formulation.

However, one of the major disadvantages hindering the use of PNiPAAm as an injectable scaffold is that PNiPAAm has a natural tendency to undergo syneresis post formation. In 2012, Ekenseair et al. showed that by using a hydrophilic, cytocompatible polyamidoamine (PAMAM) macromer as chemical crosslinker, one can counteract this phenomenon from occurring. In this work, PNiPAAM was copolymerized with glycidyl methacrylate (GMA) to form a thermogelling macromer with pendant epoxy rings that could interact
with the amine groups located on the ends of the PAMAM macromer to form a chemically crosslinked gel network. Thus, the hypothesis of our preliminary experiments was to determine if we could use these same pendant epoxy rings as active sites where functionalized magnetic nanoparticles could chemically bind to the polymer structure, and thereby, endow the nanocomposite hydrogel with unique magnetic properties that permit spatiotemporally-controlled signaling using an external magnetic field (Fig. 20).

Figure 20: Synthesis and formation of injectable thermosensitive magneto-responsive hydrogels

The results presented in this section report on the synthesis and characterization of a novel class of injectable, thermally and chemically dual-gelling bionanocomposite hydrogels from thermogelling pNiPAAm-based macromers, degradable PAMAM-based crosslinking macromers, and functional hybrid Fe$_3$O$_4$ NPs. These nanocomposite hydrogels can respond to external magnetic fields and potentially be used to guide the regenerative process of damaged articular cartilage \textit{in vivo}. The efficacy of \textit{in situ} hydrogel formation, dimensional stability, mechanical properties, reaction kinetics, tangential force measurements, and
viability of encapsulated mesenchymal stem cells were evaluated; and the effects of polymer and NP chemistry and loading were investigated.

3.2 Materials and Methods

**Materials.** N-isopropylacrylamide (NiPAAm), glycidyl methacrylate (GMA), 2,2’-azobis(2-methylpropionitrile) (azobisisobutyronitrile, AIBN), N,N’-methylenebisacrylamide (MBA), piperazine (PiP), 4-methoxyphenol (inhibitor), sodium hydroxide, and diethyl ether were purchased from Sigma-Aldrich (Sigma, St. Louis, MO), and used as received. Ethanol was purchased from Decon Labs (King of Prussia, PA) and used as received. Dimethylformamide (DMF) from Macron Fine Chemicals (Center Valley, PA), 1,4 Dioxane from Alfa Aesar (Ward Hill, MA), and HPLC Grade Acetone and Methanol from Sigma-Aldrich were purchased and dried with molecular sieves for 24 hours prior to the thermogelling macromer synthesis and purification discussed below. Dulbecco’s phosphate-buffered saline (PBS) solution was mixed from powder (pH 7.4, Gibco Life, Grand Island, NY), and ultrapure water was obtained from a Thermo Scientific Barnstead nanopure ultrapure water purification system (ThermoFisher Scientific Inc., Cambridge, MA). Amine-functionalized 50 nm and 500 nm iron oxide (Fe₃O₄) NPs and 40 nm non-functionalized NPs were purchased from Ocean Nanotech, LLC (San Diego, CA) and were diluted to specified concentrations by adding PBS solution. The size of the NPs was confirmed by high-contrast transmission electron microscopy (TEM) (JEOL JEM-1010, Peabody, MA).

**Thermogelling Macromer (TGM) Synthesis.** The thermogelling macromer p(NiPAAm-co-GMA) was synthesized by free radical polymerization as previously reported. ³³ 30 g of the comonomers, NiPAAm and GMA at 92.5 and 7.5 mol %, respectively, were dissolved
in 300 mL of either DMF or 1,4 Dioxane and polymerized at 65 °C under a nitrogen atmosphere. AIBN was added as a free radical initiator at 0.7 mol % of the total monomer content, and the reaction mixture was continuously stirred for 16 h. 0.3 g of 4-methoxyphenol inhibitor was added to the reaction mixture to ensure that the chemical reaction had ended. The product was concentrated by rotary evaporation, dissolved in a mixture of 95% acetone and 5% methanol, and twice precipitated in 10 times excess of cold diethyl ether to remove unreacted components and low molecular-weight oligomers. The final filtrate was dried under vacuum to yield a fine white powder.

**Polyamidoamine Synthesis.** The polyamidoamine (PAMAM) cross-linking macromers were synthesized by polyaddition of PiP with MBA following reported protocols. 10.83 g of the comonomers were dissolved in 30 mL of deionized water with a stoichiometric excess of MBA \( r = [\text{MBA}] / [\text{PiP}] = 0.78 \), stirred continuously under nitrogen atmosphere at room temperature, and allowed to react for 5h. The obtained viscous mixture was directly precipitated in 10 times excess of cold acetone, filtered, and dried under vacuum to yield a fine white powder.

**Proton Nuclear Magnetic Resonance (H NMR).** \(^1\)H NMR spectra were obtained using a 500 MHz spectrometer (Varian Inova, California). Samples were dissolved in D\(_2\)O (typical concentration: 20 mg/mL) that contained 0.75 wt. % 3-(trimethylsilyl) propionic-2, 2, 3, 3-d\(_4\) acid, sodium salt (TSP) as internal shift reference (Sigma-Aldrich, St. Louis, MO) and the compositions were calculated according to reported protocols.

**Gel Permeation Chromatography (GPC).** Molecular weight distributions of p-(NiPAAm\(_{92.5-co}\)-GMA\(_{7.5}\) polymers were determined with a TOSOH HLC-8320 GPC EcoSEC system (King of Prussia, PA) in mobile phase at 30°C. Samples were prepared in
a solution of 80% methanol-20% water at a concentration 1 mg/mL and were filtered prior to analysis. Macromer number-average molecular weight ($M_n$), weight average molecular weight ($M_w$), and polydispersity index (PDI) were determined in triplicate relative to polyethylene glycol standards.

**Nanocomposite Hydrogel Formation.** Individual solutions of the TGM and PAMAM macromers and Fe$_3$O$_4$ NPs were prepared. 500 µL of a 30 wt.% TGM solution, 250 µL of a 21 wt.% PAMAM solution and 250 µL of the Fe$_3$O$_4$ NPs suspension (1.25 or 2.5 mg/mL) were mixed for ~ 30 s at 4 °C in a vial to make 1 mL of the injectable hydrogel solution (Fig. 20). The composition was then injected into Teflon molds (8 mm diameter, 2 mm height) at 37 °C using cold pipet tips and the hydrogels were allowed to chemically and thermally crosslink for 2 hours. PBS was added to hydrogels with no NPs to keep the concentrations of TGM and PAMAM in our experimental control hydrogels constant.

**Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy (SEM/EDX).** Hydrogel solutions were made in Teflon molds and kept for 24 hours in a -20 °C environment and subsequently lyophilized overnight. Samples were then sectioned, coated with 3.5 nm of platinum and analyzed under high-resolution field emission scanning electron microscopy (SEM) using a Hitachi S-4800 (Hitachi High Technologies, Northridge, CA).

**Differential Scanning Calorimetry (DSC).** The LCSTs of the nanocomposite hydrogels were determined by DSC. Solutions were pipetted into aluminum volatile sample pans (TA Instruments, Newcastle, DE) and capped/crimped and allowed to stand in the incubator for 3 hours. Thermograms were recorded in triplicate on a TA Instruments DSC Q2000 with a refrigerated cooling system against an empty hermetically sealed pan as a reference. In a
typical run, the oven was equilibrated at 5°C for 5 min and then heated up to 60°C at a rate of 5°C/min. Both the LCST onset and peak temperature were determined using the Universal Analysis 2000 software provided with the DSC system.

**Rheological Characterization of Gelation Kinetics.** Rheology was used to determine the gelation kinetics of the nanocomposite hydrogel scaffolds. Injectable hydrogel formulations were pipetted onto a TA Instruments Discovery HR1 hybrid rheometer, and the dynamic viscoelastic properties of the solutions were determined, namely, the dynamic shear storage (G’) and loss (G’’ ) moduli, by utilizing a 40-mm cone (1 degree) with a gap size of 27 μm. In a typical run, the sample was placed on the rheometer Peltier plate, equilibrated at 4 °C for 5 min, heated up to 37 °C at a rate of 8 °C/min, and held constant at 37 °C for approximately 3 hours.

**Superconducting Quantum Inference Device (SQUID) Magnetometer.** Different hydrogel test groups at NP loadings of 0, 313, 625, 1250, and 2500 µg/mL were formed in vials immersed in a water bath at 37 °C for 3 hours to ensure thermal and chemical gelation occurred. The hydrogels were then put into small plastic capsules analyzed in a Quantum Design MPMS XL-5 SQUID Magnetometer (Quantum Design, San Diego, CA) with a 5 tesla maximum field. The temperature was kept constant at 37 °C (310 K), and the magnetic field was swept from -5 to 5 tesla. From the data, the magnetic susceptibility, moment and saturation of the nanocomposite hydrogels were determined.

**Tangential Magnetic Response Force Measurements.** In theory, to evaluate the magnetic forces on a single particle idealized as a magnetic sphere of uniform density, the overall response of a magnetic particle in a fluid to a magnetic field determined by the
strength and gradient of the applied magnetic field was calculated according to equation 1 (pg. 33):

\[
\vec{F}_m = V_p \Delta \chi \nabla \left( \frac{\vec{B}^2}{2\mu_0} \right) \quad \text{(Eq. 1)}
\]

where:
- \( \vec{F}_m \) = magnetic force
- \( V_p \) = volume of nanoparticle
- \( \chi \) = volumetric magnetic susceptibility (obtained from SQUID analysis)
- \( \vec{B} \) = applied magnetic field
- \( \mu_0 \) = permeability to vacuum (constant) = \( 4\pi \times 10^{-7} \text{Tm}^{-1} \)

Equation 2 can be expanded in explicit form to yield

\[
\vec{F}_m = V_p \Delta \chi \nabla \left( \frac{\vec{B}^2}{2\mu_0} \right) = \frac{V_p \chi_p}{\mu_0} \left[ B_x \frac{\partial B_x}{\partial x} + B_y \frac{\partial B_y}{\partial y} + B_z \frac{\partial B_z}{\partial z} \right]
\]

This equation can further be simplified by assuming that the magnetic field gradient changes only in the z-direction if a permanent magnet is placed directly under the hydrogel which was the case in the tangential force measurements experiments. Thus, changes in the magnetic field in the x and y plane are negligible \( (\partial B_x = \partial B_y = 0) \). This can be further simplified to

\[
\vec{F}_m = \frac{V_p \chi_p}{\mu_0} \left[ B_z \frac{\partial B_z}{\partial z} \right].
\]
The magnetic susceptibility of the hydrogel was determined through SQUID analysis, and
by knowing the maximum magnetic field generated at the surface of the magnet, we could
generate a magnetic field map that correlated the magnetic field strength relative to the
distance from the surface of the magnet. Assuming that the nanoparticles are uniformly
distributed throughout the hydrogel, we can calculate the magnetic field strength that each
particle is experiencing in the hydrogel, and thus calculate the magnetic force generated.
The sum of all the magnetic forces generated on each particle incorporated into the
hydrogel equals the total force experienced by the gel at any given distance. This approach
was used to develop a theoretical model that could be compared with the results from the
tangential force measurements.

The quantification of the magnitude of tangential forces on the nanocomposite hydrogels
were also determined. Hydrogels were placed in a plastic basket and hung from the base of
an analytical balance. Rare earth neodymium magnets (N42) were placed on a mechanical
stage and gradually raised toward the hydrogel. The distance from the base of the basket
to the tip of the magnet was recorded. Increases in the weight of the hydrogel was equated
to the total magnetic force exerted by the NPs in the hydrogel network. The experiment
was replicated 7 times for each hydrogel test group.

**Mesenchymal Stem Cell (MSCs) Cell Encapsulation.** MSCs were harvested from the
femora and tibiae of 6−8 week old male rats following established procedures in
accordance with approved protocols by Northeastern University. The rats were
euthanized by CO₂ asphyxiation and a bilateral thoracotomy. The tibiae and femora were
aseptically removed, placed in Dulbecco’s modified Eagle’s media (DMEM) (Sigma, St.
Louis, MO) with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 3%
penicillin–streptomycin–fungizone (Invitrogen), and flushed with modified eagle-alpha media (α-MEM) with 10% FBS, 10mM b-glycerol-2-phosphate, 50 mg/L ascorbic acid, and 1% penicillin–streptomycin–fungizone (Invitrogen). The marrow pellets were broken up, sterile-filtered, and plated in 75 cm² tissue culture flasks at 37°C under humidified, 5% CO₂ atmosphere with the modified α-MEM media for 6 days. The TGM and PAMAM polymers as well as the Fe₃O₄ NPs were UV-sterilized for 24 h. Prior to the experiment the TGM polymers were dissolved overnight in osteogenic media and the PAMAM polymer was dissolved in media just before the experiment was conducted.

MSCs were put into single cell suspension by trypsinization, and were washed, counted, and resuspended in osteogenic media just prior to being added into the injectable nanocomposite hydrogel solution. Stem cells were added at a cell density of 1.25 million cells/mL to bionanocomposite hydrogels with 50 nm and 500 nm amine-functionalized NPs to obtain a final concentration of 625 μg of NPs/mL of hydrogel. The solutions were manually mixed, pipetted into 24 well plates, allowed to cross-link at 37 °C in an incubator for 1 hour, immersed with 2 mL media, and cultured for 7 days (changing the media every day). The bionanocomposite hydrogels were then removed and washed in PBS solution to remove the growth media. Samples were incubated with 1 mL of 1 μM Live Green and 1mM Dead Red per the manufacturer’s instructions (Live/Dead Cell Imaging Kit (488/570), Thermo Fisher Scientific). The hydrogels were then imaged under a fluorescent microscope (Zeiss Observer Z1, Germany) using a 10x objective to visualize the encapsulated cells in the hydrogel structure where the live cells gave a bright green fluorescence and dead cells gave a bright red fluorescence. Cell viability was quantified by
counting live and dead cells using ImageJ software. Percent viability was calculated as the number of live cells divided by the total number of cells (live+dead) (n=6-9).

Statistics. Results were expressed as means ± standard deviation. Data analysis was conducted using Prism 7 statistical software (GraphPad, La Jolla, CA, USA). For significance, a minimum p-value of <0.05 was used. For comparison of two mean values, an independent variable t-test was performed. For comparison of three or more means, a one-way analysis of variance (ANOVA) was performed.

3.3 Results

Polymer Characterization

A thermosensitive hydrogel scaffold was created by copolymerizing pNiPAAm with GMA in a free radical polymerization reaction to develop a TGM with pendant epoxy rings, as reported in previous research. The copolymer composition of the TGM was confirmed to be 92.5 mol.% NiPAAm and 7.5 mol.% of GMA by 1H NMR (data shown in the Appendix section, Fig 48.). The molecular weight of the TGM formed can be slightly controlled by the solvent used in the free radical polymerization procedure. In this research, two different solvents, namely DMF and 1,4-dioxane, were used in the making of the TGM to achieve two different molecular weights. The molecular weight distribution of the synthesized TGM made in both solvents was determined by Gel Permeation Chromatography (GPC). The data from the GPC showed that polymers made in DMF had a number average molecular weight, $M_n$, of 9 kDa with a polydispersity (PDI) of 2.11, while polymers made in 1,4 Dioxane had an $M_n$ of 21 kDa (PDI of 2.66).
Next a cytocompatible chemical cross-linking macromers, PAMAM, were synthesized according to adapted published protocols. The PAMAM macromers were created by the polyaddition of PiP and MBA at room temperature. The synthesis of PAMAM was confirmed by $^1$H NMR (data shown in Appendix, Fig. 49). From the $^1$HNMR graph, an experimental average molecular weight of the PAMAM macromer was calculated to be ~ 2100 Da. The addition of these chemical crosslinkers into the hydrogel network not only enabled tuning the degree of swelling but also controlling the degradation rate (via hydrolysis) of the hydrogel scaffold to match the rate of tissue growth.

Figure 21: Gel Permeation Chromotography (GPC) results showing the molecular weight distribution of TGMs made in 1,4 Dioxane and DMF.
Nanoparticle Functionalization

One of the key advantages of using our hydrogel system is the fact that the gel system undergoes a dual physical and chemical gelation mechanism. The thermal transition of NiPAAm from room temperature to body temperature rapidly occurs, and the PAMAM macromers and amine-functionalized iron oxide NPs act as active chemical crosslinkers forming a gel network within the thermogelling macromers.

In an initial effort to incorporate the MNPs into the hydrogel scaffold, Fe$_3$O$_4$ (40 nm and 500 nm) were purchased and functionalized in house with a ligand called 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) or DSPE-PEG Amine. DSPE=PEG Amine has a hydrophobic end that interacts with the surfactant on the surface of the MNPs, and a hydrophilic amine end that creates hydrogen bonds with surrounding water molecules and chemical crosslinks with the epoxy rings located on the TGM backbone. With this process, different nanocomposite hydrogel scaffolds were created (Fig.22).

Figure 22: A picture showing a hydrogel without any MNPs (left), a hydrogel with DSPE-PEG Amine functionalized 40 nm particles incorporated into the hydrogel scaffold (middle), and a hydrogel with DSPE-PEG Amine functionalized 500 nm particles incorporated into the hydrogel scaffold
Rheology was used to monitor the changes in $G'$ and $G''$ and the results of the experiments are shown in the Appendix (Fig. 50). The results of the experiment seem to suggest that perhaps the introduction of the DSPE-PEG Amine ligand into our hydrogel formulation may be affecting the chemical gelation process of our hydrogel system after the initial thermogelation process. Due to this result, our lab moved to purchase already amine-functionalized MNPs (50 nm and 500 nm) from Ocean Nanotech, LLC.

**SEM/EDX Analysis:**

![SEM images](image)

Figure 23: Cross-sectional SEM images of nanocomposite hydrogels with 50 nm (a and b) and 500 nm (c) Fe3O4 NPs at a loading of 625 µg/mL. EDX analysis of the surface of a hydrogel near (d) and far (e) from the NPs.

SEM images of nanocomposite hydrogel samples were taken along with EDX analysis confirmed the presence of Fe$_3$O$_4$ NPs on the hydrogel surface (Fig. 23). The SEM images suggest that there is an even distribution of NPs throughout the hydrogel. Even distribution of NPs within the scaffold is important to initiate physical changes throughout the hydrogel so that encapsulated cells experience changes to their local microenvironment when the scaffolds is subjected to an external magnetic field. This type of stimulation could lead to
the activation of various mechano-transduction signaling pathways that could induce osteogenic stem cell differentiation.\textsuperscript{85}

**LCST Studies:** To evaluate the effect of the incorporation of Fe\textsubscript{3}O\textsubscript{4} NPs in the nanocomposite hydrogel scaffolds, DSC analysis of the gels were performed. Literature informs us that the LCST of PNiPAAm is \(~32\) °C.\textsuperscript{122} From the data shown on the graph (Fig. 24), it appears that the incorporation had little or no effect on the LCST of the thermogelling hydrogel, thereby suggesting the feasibility of using nanocomposite hydrogels as injectable scaffolds \textit{in vivo}.

![Figure 24: Effect of NP incorporation on the LCST of pNiPAAm-based nanocomposite hydrogels. No statistical difference was determined between the test groups by one-way ANOVA; p < 0.05.](image)

**Gelation Kinetics:** Once more, rheology was used to monitor the changes in \(G'\) and \(G''\) and the results of the experiments are shown in Fig. 25. The chemical crosslinking in the nanocomposite hydrogels increased the shear storage modulus of the hydrogel system by
3 orders of magnitude leading to an ultimate shear storage modulus ranging from 0.5 to 1 MPa for all experimental conditions.

![Graph showing storage modulus over time with different NP loadings and sizes.](image)

Figure 25: Oscillatory rheology traces showing dual thermal and chemical gelation for injectable nanocomposite hydrogel solutions with 15 wt % TGM, 5 wt % PAMAM and NP loadings of 625 µg/mL of either 50 nm or 500 nm NPs.

**Magnetic Properties of Nanocomposite Hydrogels:**

![Graphs showing magnetic properties.](image)

Figure 26: SQUID magnetometer analysis of nanocomposite hydrogels showing the effect of NP size and loading on magnetic behavior of scaffolds (a). Values for magnetic saturation and magnetic susceptibility were obtained from the graphs b and c, respectively.
Addition of the superparamagnetic NPs into the hydrogel structure endowed the nanocomposite scaffolds with unique magnetic properties that could be characterized by using a Superconducting Quantum Interference Device (SQUID) (Figure 26). The magnetic susceptibility is a measure of the extent to which a substance can be magnetized when subjected to an external magnetic field, and the saturation magnetization is the state reached by magnetic materials where increases in the magnetic field do not increase the magnetization of the material any further. From the data shown in figure 5a, it suggests that the nanoparticles can maintain the superparamagnetic behavior after incorporation into the hydrogel backbone. Furthermore, we observed that, NP loading on samples containing NPs of the same size had a significant effect on the saturation magnetization response in the SQUID measurements. At NP loadings below 1000 µg/mL, the saturation magnetization was higher in the nanocomposite hydrogels with 500 nm NPs, while at higher loadings (> 1000 µg/mL) the saturation magnetization is higher in the nanocomposite hydrogels with the smaller 50 nm NPs (figure 5b). The data show that, nanocomposite hydrogels with smaller NPs generally have a higher magnetic susceptibility than hydrogels with larger NPs (figure 5c). Moreover, the SQUID data confirmed that, as the magnetic field goes through 0 (no magnetic field), the hydrogels showed no magnetic response, suggesting that, the nanocomposite hydrogels exhibit a superparamagnetic behavior.

**Tangential Force Measurements:**

Tangential measurements were made to determine the amount of magnetic force that can be applied on the nanocomposite hydrogels (Fig. 27). It was experimentally determined that by using a low magnetic field of 0.5 tesla, engineering stresses of 52.5 and 29.6 Pa
could be generated for hydrogels with 625 μg/mL of 50 and 500 nm NPs, respectively. This means that engineering strains of 2.62 and 1.48 % are achievable in our hydrogel samples containing a loading of 625 μg/mL of 50 and 500 nm NPs. The concentration of iron oxide NPs is a considerable factor in manipulating the engineering stress applied on the cells given a known magnetic field.

Figure 27: Experimental and theoretical tangential forces generated by placing nanocomposite hydrogels in a 0.3 tesla magnetic field.

Possible reasons for discrepancies between actual vs. theoretical results could be due to the assumption that was made in the mathematical model that the NPs in the hydrogel do not interact with one another. However, when a magnetic field is induced on the hydrogel, the NPs generate their own magnetic fields, which can interact with other nearby NPs especially if there is agglomeration of NPs within the hydrogel. The NPs would be pulled towards the agglomeration, thereby taking away the full effect of the force over the entire hydrogel. Furthermore, since the NPs are chemically crosslinked into the polymer backbone, the restricted movement of the NP diminishes the amount of force exerted by
the particle in the hydrogel. However, a simple solution to overcome this limitation may be to either increase the concentration of NPs in the hydrogel, or increase the magnetic field strength. The Young’s modulus of the hydrogel system was previously reported to be around 2 kPa in literature. With this information, the engineering stress and strain can be determined by using Hooke’s Law.

**Mesenchymal Cell Encapsulation Studies:** Several studies have been performed to investigate the toxicity of Fe$_3$O$_4$ NPs on cells. However, in our hydrogel stem, because the amine-functionalized NPs are actually being chemically bonded to the polymer backbone through an epoxy-amine reaction, the toxic effect of the NPs on the encapsulated cell populations is significantly reduced. Mesenchymal stem cells harvested from 6-8 week-old rats were encapsulated into nanocomposite hydrogels with 50 nm and 500 NPs at a loading of 625 µg/mL for 7 days and cell viability was determined by fluorescent microscopy (Fig. 28).

![Fluorescent microscopy images](image)

Figure 28: Fluorescent microscopy images of live/dead (green/red) stained hydrogels with encapsulated MSCs after 7 days of culture without NPs (a), with 625 µg/mL 50 nm NPs (b), and with 625 µg/mL 500 nm NPs (c).
Figure 29: MSC viability over a 7 day period without NPs or with 625 µg/mL of either 50 nm or 500 nm NPs. Statistical differences were seen between experimental groups were analyzed by one-way ANOVA, and differences within the same group over different days were analyzed by t-tests; * (p ≤ 0.05)), ** (p ≤ 0.01)), *** (p ≤ 0.001)), **** (p ≤ 0.0001)).

From this study, it was determined that MSCs could remain viable up to seven days in the bionanocomposite hydrogel environment. While there was an initial trend towards higher cell viability when 500 nm nanoparticles were encapsulated, overall no statistical differences in cell viability were detected between groups (Figure 29) indicating that the chemically-conjugated incorporation of the iron oxide nanoparticles into the hydrogel network structure did not adversely impact the encapsulated stem cells. Overall, within the test groups, there were statistical differences in the fluorescent images of cells recorded from day one to day seven for the control group and across each time point for the bionanocomposite hydrogels with 50 nm NPs. These results demonstrated that it is possible to deliver viable stem cell populations within our injectable, magneto-responsive hydrogel scaffold system.
3.4 Discussion

Biomaterials that can respond to physical changes within their local environment show great promise as active scaffolds for tissue engineering and regenerative medicine purposes. In recent literature, magneto-responsive biomaterials have been used as scaffolds for bone,[31, 32] cartilage,[29, 33] cardiovascular[34] and neuronal[35] tissue regeneration applications. In terms of cartilage tissue regeneration specifically, a constant challenge in the field is the inability to recreate the multiple cartilage layers found in native cartilage. However, magneto-responsive scaffold could provide a solution to this challenge. By subjecting the magneto-responsive hydrogel to a strong magnetic field gradient, one can create a variation in force distribution within the scaffold in which encapsulated stem cells can respond to theoretically form different layers of cartilage.

The data presented above proves that our hydrogel system is injectable (minimally-invasive), responsive to a magnetic field, and able to maintain stem cell populations for over several days. Furthermore, we have shown that we can easily manipulate magnetic properties of the nanocomposite hydrogels by changing NP size, loading or magnetic field strength. However, more work needs to be done to develop better theoretical models to be able to accurately predict the magnetic force generated by the scaffold at a given magnetic field.

3.5 Conclusion

In conclusion, an injectable hydrogel was made by combining a thermoresponsive macromer copolymerized with GMA (p(NiPAAm92.5-co-GMA7.5)) along with a hydrophilic PAMAM chemical crosslinker. 1H NMR and GPC were used to confirm polymer composition and determine molecular weight distributions. Amine-functionalized
NPs (50 nm and 500 nm) were chemically incorporated into the hydrogel backbone by an epoxy-amine reaction. SQUID magnetometer analysis indicated that NP loading and size have a significant effect on hydrogel response to an external magnetic field. Moreover, tangential force measurements quantified the amount of force that could be generated within the hydrogel scaffold under a static magnetic field. And at low magnetic field strength of 0.3T, physiologically-relevant engineering stresses of 52.5 and 29.6 Pa were generated for hydrogels with 625 μg/mL of 50 or 500 nm NPs, respectively. Finally, primary MSCs were encapsulated within the nanocomposite hydrogel up to seven days. The inclusion of the nanoparticles had no significant impact on MSC viability. The results demonstrated above show that these magnetic hydrogels could be used as injectable scaffolds that can allow real-time spatiotemporal control of deformation of the hydrogel which would lead to the physical stimulation of encapsulated cells. Furthermore, the MSC cytocompatibility study suggests that the chemical conjugation of the NPs into the hydrogel structure could effectively limit the cytotoxic effects of the iron (III) oxide nanoparticles on neighboring cells.

3.6 Recommendations/Future Directions

To progress the work presented in the research presented in the first aim of this thesis, the author has a few suggestions on how what future directions that could be taken to extend the work already conducted.

- **Bioresorability:** One of the main challenges with using PNiPAAm-based scaffolds for tissue engineering applications is that PNiPAAm is not biodegradable in the body. However, to address the issue of non-degradability of PNiPAAm based hydrogels, many research groups have attempted to enhance bioresorability of
PNiPAAm polymers by copolymerizing the complex with pendant groups such as dimethyl-γ-butyrolactone acrylate (DBA), a hydrolysable pendant lactone ring or polyester side groups that modulate the LCST over time via hydrolysis.\textsuperscript{64} It would be interesting to investigate the possibility of incorporating DBA into our TGM polymer chain backbone to tune the bioresorable properties of our hydrogel platform.

**In Vivo Biocompatibility:** One of the experiments that we were not able to conduct was an *in vivo* study on the biocompatibility of our hydrogel scaffold. In this proposed experiment, a subcutaneous injection of our hydrogel formulation would be placed directly into small animal model to investigate if our nanocomposite hydrogel initiates any adverse immune response in the host body. Furthermore, we would like to see if over time the MNPs would detach from the TGM backbone, migrate and deposit themselves in other vital organs/tissues in the body.

**Optimization of computational models to better predict magneto-responsive biomaterial behavior *in vivo***: An ongoing challenge in the field of magneto-responsive biomaterials is that it is difficult to accurately predict the behavior of the magnetic scaffold in the presence of an external magnetic field. In the research presented in Aim 1, we attempted to advance this area of the field by developing a mathematical model to determine the force distribution within our hydrogel scaffold using a series of differential equations. We compared our mathematical model to the results obtained in our tangential force measurements and we found out that our experimental force results were much lower than our calculated values. We speculate that some reasons for these discrepancies between actual vs.
theoretical results could be due to the assumption that was made in the mathematical model that the NPs in the hydrogel do not interact with one another. However, we know that when a magnetic field is induced on the hydrogel, the NPs generate their own magnetic fields, which can interact with other nearby NPs. Furthermore, since the NPs are chemically crosslinked into the polymer backbone, the restricted movement of the NP diminishes the amount of force exerted by the particle in the hydrogel. If we could combine these restrictions mathematically into our theoretical model perhaps our predicted values could align more with our experimental values.
Chapter 4: Fabrication of Porous, Injectable Hydrogel Scaffolds for Bone and Cartilage Repair (Research Aim 2)
4.1 Introduction

In order to regenerate a heterogeneous complex tissue structure, cellular infiltration into the scaffold must be efficient. Scaffolds that have a highly porous structure do not only allow for uniform cellular distribution throughout the scaffold, but also promote good integration with the host tissue, therefore, making porosity a quintessential factor for scaffolds used to repair osteochondral defects. The optimum pore size required for cells to freely migrate varies depending on the cell’s phenotype, however, the consensus in literature is that a minimum size of 100 µm is compulsory for proper cellular migration and the vascularization of the ingrown tissue. A variety of techniques, such as particulate leaching and gas foaming, have been utilized to increase porosity within injectable scaffolds. Unfortunately, these techniques often involve the use of toxic organic solvents that could have adverse effects on cell viability.

An attractive alternative to this ongoing research challenge is to incorporate degradable micro-particles as porogens into an injectable biomaterial formulation pre-solidification. Liao et al. (2011) conducted a comparative study to investigate bone response to an injectable calcium phosphate with different polymeric microspheres made of either poly(lactic-co-glycolic acid) (PLGA), gelatin or poly(trimethylenecarbonate) (PTMC)) as porogens to increase macroporosity within injectable scaffolds. From their results, they found that composite calcium phosphate scaffolds with PLGA microspheres showed a significantly better bone response than composite scaffolds with gelatin or PTMC microspheres.

However, other studies have advocated for the use of gelatin microspheres as porogens within injectable scaffolds as gelatin is a natural biomaterial that is biodegradable and
confers very low antigencity.\textsuperscript{131} Gelatin is a highly biocompatible and biodegradable material derived from collagen and has been widely investigated as a drug delivery carrier due to its adaptable drug loading and release kinetics which can be independently optimized by tuning the material’s molecular weight or crosslinking density.\textsuperscript{132} The Mikos lab at Rice University has shown that an added benefit of using gelatin microspheres as porogens in injectable scaffolds is that you can utilize these unique microspheres also as drug and cell delivery carriers within the scaffold.\textsuperscript{132} For example, in articles published from this lab circa 2003-2005, they observed an increase in in cellular proliferation when they released transforming growth factor-b1 (TGF-β1) from gelatin micro-particles incorporated into oligo(poly(ethylene glycol) fumarate) (OPF) injectable scaffolds.\textsuperscript{133-134} The results showed a statistically significant increase in glycosaminoglycans (GAGs) content over 4 weeks in samples with TGF-β1 loaded in the gelatin micro-particles. Furthermore, gelatin microspheres have also been used to shield cells during the crosslinking process of an injectable poly(propylene fumarate) (PPF) scaffold.\textsuperscript{135-136} This duality of benefits as a porogen and cell/drug delivery carrier make gelatin micro-particles a viable option to increase scaffold macro-porosity and cellular infiltration within injectable scaffold. In this project, the feasibility of increasing the macro-porosity within the magneto-responsive biomaterial (developed in Aim 1). The effect of micro-particle size on hydrogel formation, dimensional stability, magnetic properties, reaction kinetics, and cellularity evaluated. Furthermore, the feasibility of using magnetic field to accelerate the release of a therapeutic agent from the GMP-composite magnetic hydrogels was investigated.
4.2 Materials and Methods

Materials. *N*-isopropylacrylamide (NiPAAm), glycidyl methacrylate (GMA), 2,2’-azobis(2-methylpropionitrile) (azobisisobutyronitrile, AIBN), *N,N*-methylenebisacrylamide (MBA), piperazine (PiP), 4-methoxyphenol (inhibitor), collagenase (enzyme) and diethyl ether were purchased from Sigma-Aldrich (Sigma, St. Louis, MO), and used as received. 10,000 MW Dextran, manufactured by Pharmacosmos, Denmark, was donated by the Webster Group at Northeastern University. HPLC Grade Acetone and Methanol from Sigma-Aldrich were purchased, dried with molecular sieves for 24 hours prior to the thermogelling macromer synthesis, and purified as discussed below. Dulbecco’s phosphate-buffered saline (PBS) solution was mixed from powder (pH 7.4, Gibco Life, Grand Island, NY), and ultrapure water was obtained from a Millipore Super-Q water system (Millipore, Billerica, MA). Amine-functionalized 500 nm iron oxide (*Fe₃O₄*) NPs were purchased from Ocean Nanotech, LLC (San Diego, CA) and were diluted to specified concentrations by adding PBS solution.

**Thermogelling Macromer (TGM) Synthesis.** The thermogelling macromer p(NiPAAm-co-GMA) was synthesized by free radical polymerization as previously reported. Briefly, 30 g of the comonomers, NiPAAm and GMA at 92.5 and 7.5 mol %, respectively, was dissolved in 300 mL of 1,4 Dioxane and polymerized at 65 °C under a nitrogen atmosphere. AIBN was added as a free radical initiator at 0.7 mol % of the total monomer content, and the reaction mixture was continuously stirred for 16 h. 0.3 g of 4-methoxyphenol inhibitor was added to the reaction mixture to ensure that the chemical reaction had ended. The product was concentrated by rotary evaporation, dissolved in a mixture of 95% acetone and 5% methanol, and twice precipitated in 10 times excess of cold diethyl ether to remove
unreacted components and low molecular-weight oligomers. The final filtrate was dried under vacuum to yield a fine white powder.

**Polyamidoamine Synthesis.** The polyamidoamine (PAMAM) cross-linking macromers were synthesized by polyaddition of PiP with MBA following reported protocols.\(^{63}\) 10.83 g of the comonomers was dissolved in 30 mL of deionized water with a stoichiometric excess of MBA \((r = [\text{MBA}] / [\text{PiP}] = 0.78)\), stirred continuously under nitrogen atmosphere at room temperature, and allowed to react for 5 h. The obtained viscous mixture was directly precipitated in 10 times excess of cold acetone, filtered, and dried under vacuum to yield a fine white powder.

**Gelatin (GMP) Micro-particles Synthesis.** GMPs were synthesized using a procedure adapted from published protocols.\(^{134,137}\) To synthesize 50-400 µm micro-particles, 5.0 g of bovine gelatin (Sigma-Aldrich, St. Louis, MO) was dissolved into 45 mL ddH2O. Dissolution was achieved by means of stirring the solution intermittently while keeping it at 70°C. The resulting solution was added dropwise to a mixture of 250 mL olive oil and 1.25 mL Span 80 (Sigma-Aldrich, St. Louis, MO), which was mixed at 500 rpm using an overhead stirrer. The reaction vessel was surrounded by ice and the emulsion was allowed to continue to mix for 30 min, at which point 100 mL of chilled acetone was added dropwise to the emulsion. After an additional 1 h of mixing, the formed micro-particles were filtered out and washed with acetone. Crosslinking of the micro-particles with 10 mM glutaraldehyde (Sigma-Aldrich, St. Louis, MO) was achieved by incubating them in a solution of 0.5 mL Tween 80 (Sigma-Aldrich, St. Louis, MO) in 500 mL ddH2O with 1 g glutaraldehyde while stirring at 500 rpm and keeping the reaction vessel surrounded by ice. The crosslinking reaction was terminated after 20 h by adding 0.9375 g glycine (Sigma-
Aldrich, St. Louis, MO) to the reaction vessel and allowing the mixture to stir for 1 h. The crosslinked micro-particles were isolated by filtering and washing with chilled water and acetone. Micro-particles were frozen in a -20°C freezer for at least 1 h prior to overnight lyophilization. Particles were sieved to collect those with diameters between 120 and 230 µm and between 230 and 400 µm. Morphology and size of GMPs for a given distribution were confirmed using Scanning Electron Microscopy (Hitachi S-4800, Hitachi High Technologies, Northridge, CA). Before imaging, GMPs were placed on an adhesive SEM stage and sputter-coated with 5.0 nm platinum.

**Formation of micro-particle hydrogels:** To prepare GMPs for incorporation into the hydrogel, the micro-particles were pre-swollen in PBS or media at a ratio of 5 mL PBS to 1 g GMP ratio for 16 h. A 0.3 mg/mL TGM solution was prepared by dissolving TGM overnight in PBS or media. Immediately prior to hydrogel formation, PAMAM was dissolved in PBS or media to make a 0.4 mg/mL solution. For a 1 mL hydrogel, 250 µL of the PAMAM solution was first mixed with 50 mg of either 120 µm or 230 µm pre-swollen GMPs in a 4 mL plastic vial. 250 µL of the Fe₃O₄ NP suspension and 500 µL of the TGM solution were then added and mixed with the PAMAM/GMP solution to distribute the components evenly throughout the gel. In the gels that did not include nanoparticles, the NPs were replaced with 250 µL of PBS or media to keep the concentrations of each component equal across all experimental groups. The plastic vial was placed in a 37 °C water bath for 2 h.

**Scanning Electron Microscopy (SEM):** Hydrogels were dried in a six-well plate for at least 72 hours prior to preparation for SEM. Samples were then sectioned, adhered to SEM mounts, and sputter-coated with 5.0 nm platinum before imaging. Hydrogel morphology
was determined using Scanning Electron Microscopy (Hitachi S-4800, Hitachi High Technologies, Northridge, CA). Note: To accelerate the degradation of the GMPs, the hydrogels were placed in a PBS solution containing collagenase (400 ng/mL).  

**Differential Scanning Calorimetry (DSC).** The LCSTs of the micro-particle composite hydrogels were determined by DSC. Solutions were pipetted into aluminum volatile sample pans (TA Instruments, Newcastle, DE) and capped/crimped. Thermograms were recorded in triplicate on a TA Instruments DSC Q2000 with a refrigerated cooling system against an empty hermetically-sealed pan as a reference. In a typical run, the oven was equilibrated at 5 °C for 5 min and then heated up to 60 °C at a rate of 5 °C/min. Both the LCST onset and peak temperature were determined using the Universal Analysis 2000 software provided with the DSC system.

**Swelling properties:** Hydrogel samples were stored in a humidified incubator for 2 h prior to any test to allow chemical crosslinking to take place. Samples were then taken out and weighed on a balance in the formation state, placed in excess PBS for 24 h to equilibrate, reweighed in the equilibrium state, frozen at −20°C, lyophilized, and weighed a third time in the dry state. The hydrogel swelling ratios at formation, $q_{\text{formation}}$, and equilibrium, $q_{\text{equilibrium}}$, were calculated as the difference between the swollen and dry mass divided by the dry mass.

**Rheological Characterization of Gelation Kinetics.** Rheology was used to determine the gelation kinetics of the micro-particle composite hydrogel scaffolds. Injectable hydrogel formulations were pipetted onto a Discovery HR1 hybrid rheometer (TA Instruments, Newcastle, DE), and the dynamic viscoelastic properties of the solutions were determined, including the dynamic shear storage ($G'$) and loss ($G''$) moduli, by utilizing a 40 mm cone
(1 degree) with a gap size of 27 μm. In a typical run, the sample was placed on the rheometer Peltier plate, equilibrated at 4 °C for 5 min, heated up to 37 °C at a rate of 8 °C/min, and held constant at 37 °C for approximately 3 hours.

**Superconducting Quantum Inference Device (SQUID) Magnetometer.** The effect of GMP incorporation on the magnetic behavior of the hydrogel scaffold was determined by SQUID magnetometry. Different hydrogel test groups were formed in vials immersed in a water bath at 37 °C for 3 hours to ensure thermal and chemical gelation occurred. The hydrogels were then put into small plastic capsules analyzed in a Quantum Design MPMS XL-5 SQUID Magnetometer (Quantum Design, San Diego, CA) with a 5 tesla maximum field. The temperature was kept constant at 37 °C (310 K), and the magnetic field was swept from -2 to 2 tesla. From the data, the magnetic susceptibility, moment and saturation of the nanocomposite hydrogels were determined.

**In Vitro Release Studies.** GMPs were pre-swollen in a PBS solution contained 25 mg/mL of 10,000 MW Dextran before being incorporated into the hydrogel formulation. The formed hydrogels were surrounded in 3 mL of PBS and stored at 37°C. Every 30 min, PBS around the gel was collected and replaced with fresh PBS. To investigate the effect of an external magnetic field on the release profile of the dextran, samples were stored on top of a 1 tesla magnet for the duration of the experiment. The concentration of Dextran released from the micro-particle composite hydrogel was determined by a TOSOH HLC-8320 Gel Permeation Chromatography (GPC) EcoSEC system (King of Prussia, PA).

**DNA Assay.** Human Fibroblast Cells (Passage 4) were donated to us from the Webster Lab. The cells were cultured in 75 cm² tissue culture flasks at 37 °C under humidified, 5 % CO₂ atmosphere with the modified DMEM media for 7 days. The TGM and PAMAM
polymers and the Fe$_3$O$_4$ NPs were UV-sterilized for 24 h. Prior to the experiment, the TGM polymers were dissolved overnight in osteogenic media, and the PAMAM polymer was dissolved in media just before the experiment was conducted. Cells were put into single-cell suspension by trypsinization, and were washed, counted, and re-suspended in growth media just prior to being added into the injectable nanocomposite hydrogel solution. Fibroblast cells were added at a cell density of 2 million cells/mL to composite hydrogels with 500 nm amine-functionalized NPs to obtain a final concentration of 625 μg of NPs/mL of hydrogel. The solutions were manually mixed, pipetted into 96-well plates, allowed to cross-link at 37 °C in an incubator for 1 h, immersed with 2 mL media, and cultured for 14 days (changing the media every 2 days). The composite hydrogels were then removed and washed in PBS solution to remove the growth media. Samples were then collected and the DNA content in each hydrogel was determined with a Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific).

Statistics. Results were expressed as means ± standard deviation. Data analysis was conducted using Prism 7 statistical software (GraphPad, La Jolla, CA, USA). For significance, a minimum p-value of < 0.05 was used. For comparison of two mean values, an independent variable t-test was performed. For comparison of three or more means, a one-way analysis of variance (ANOVA) was performed.

4.3 Results

SEM Results: GMPs were synthesized, collected and separated into different experimental test groups by size. SEM was used to observe particle size and morphology. The average particle diameter of each test group was confirmed by SEM to be 120 μm and 230 μm respectively (Fig. 30 A&E). The GMPs from both test groups were successfully
incorporated into our composite hydrogel as seen in Fig. 30 B&F. The dual benefit of using GMPs in our hydrogel system is that not only could they serve as drug delivery carriers within our injectable, magneto-responsive hydrogel, but could also be used to increase the interconnected macro-porosity within our scaffold upon degradation, as scaffold porosity is critical for uniform cellular distribution and proliferation.

Therefore, to test this hypothesis, composite hydrogel samples were incubated in PBS containing collagenase, an enzyme secreted by mammalian cells capable of breaking down collagen (and its derivatives), to study if pores would be formed within our hydrogel upon degradation of the GMPs. The results of this experiment are seen in images C, D, G and H Fig. 30. After 5 days, we observed a partial degradation of the GMPs, and some pore sites begin to form within the scaffold. After 7 days of the collagenase treatment, almost all the GMPs have been degraded, and we observe large interconnected pores form in both particle test groups, thus confirming our hypothesis that the addition of GMPs increase macro-porosity within our composite hydrogel system.

Figure 30: SEM images showing the degradation of GMPs in the presence of collagenase (40 ng/mL) to form pores within the hydrogel over a 7-day period. (A and E) SEM images of GMPs (120 µm and 230 µm Micro-particles respectively). (B and F) GMPs incorporated into the hydrogel scaffold at Day 0. (C and G) Partial degradation of GMPs after a 5-day
exposure to collagenase. (D and H) GMPs have almost complete degraded after a 7-day exposure to collagenase creating large pores within the hydrogel scaffold.

**LCST Studies:**

The effect of GMPs incorporation on the LCST of the nanocomposite hydrogels was determined by DSC (Fig. 31). From the data presented in the graph below, it appears that the addition of the GMPs has a statistically significant effect on the thermal transition temperature of the scaffolds when compared to our experimental controls (TGM+PAMAM). However, the addition of the 500 nm nanoparticles alone did not seem to not have as much of an effect on the LCST. Possible reasons for this decrease in LCST could be because the addition of the GMPs into the hydrogel formulation increases the hydrophilic properties of the gel network thus increasing the thermogelling temperature. However, most importantly, the LCST across all test groups is still well below body temperature thereby confirming that these composite hydrogel scaffolds could be used as injectable scaffolds *in vivo.*

![Effect of Gelatin Incorporation on the LCST of Nanocomposite Scaffolds](image)

Figure 31: Effect of gelatin micro-particles size on the LCST of micro-particle composite hydrogel scaffolds (n=3). Statistical difference was determined between the test groups by one-way ANOVA; * (p ≤ 0.05), ** (p ≤ 0.01).
Swelling Properties:

Figure 32: Effect of incorporating gelatin micro-particles and magnetic nanoparticles on hydrogel swelling behavior (n=3). Statistical difference was determined between the test groups by one-way ANOVA; * (p ≤ 0.05), ** (p ≤ 0.01), *** (p ≤ 0.001).

To examine the effect of micro-particle incorporation on hydrogel swelling behavior, the weight swelling ratios of the samples at formation and after equilibrium were obtained (Fig. 32). From the data presented below shows that the addition of GMPs (both 120 µm and 230 µm) have a considerably significant effect on the swelling properties at the time of formation. However, at equilibrium, the average swelling ratios do tend to increase with the addition of micro-particles, however, the differences in swelling ratios at equilibrium in samples with micro-particles are not as great when compared to samples without micro-particles. Possible reasons for this observation could be because the GMPs are added into the injectable hydrogel solution in their swollen state increasing the initial weight of the
hydrogel. But as the samples reached equilibrium in PBS over 24 hours, samples without micro-particles were permitted to expand freely without the interference of GMPs inhibiting the full expansion of the gelled network.

**Gelation Kinetics:**
To better understand the effect that the GMPs had on the hydrogel gelation kinetics, rheology was used to monitor the changes in the viscoelastic properties of the gel (Fig. 33). Hydrogels containing larger GMPs have a higher initial shear modulus than samples with containing smaller GMPs or no GMPs at all. However, upon chemical crosslinking of the different components of the composite hydrogels, the shear storage modulus increases by 3 orders of magnitude in all experimental conditions.

![Figure 33: Effect of gelatin micro-particles size on the physical and chemical gelation mechanism of the micro-particle composite scaffolds.](image)

**SQUID Analysis:**
The magnetic behavior of composite hydrogels was tested using a SQUID magnetometer (Fig. 34). The data shows that the addition of GMPs into the scaffold diminishes the magnetic properties of our magneto-responsive scaffold. The SQUID data shows that the size of micro-particles used in the hydrogel formulation has a direct impact on the magnetic
saturation of our hydrogel scaffolds when compared to our experimental controls of hydrogels with MNPs but no GMPs. A possible explanation for this is that by adding the micro-particles into the hydrogel, the concentration of the NPs in the hydrogel scaffold is being significantly decreased.

![SQUID Analysis Graph](image)

Figure 34: SQUID magnetometer analysis of composite hydrogels showing the effect of GMP size on magnetic behavior of scaffolds.

**In Vitro Drug Release:** In this experiment, the feasibility of utilizing a composite magneto-responsive biomaterial as a controlled drug delivery platform was investigated. Dextran (MW 10,000) was used a model drug for our drug release experiment and was loaded into our 230 \( \mu \text{m} \) gelatin microspheres and were then incorporated into our magnetic hydrogel formulation. The efficacy of using this hydrogel system to deliver small therapeutic agents on-demand in the presence of a magnetic field *in vitro* was determined (Fig. 35).

Our findings show that, even in the presence of a moderately strong magnetic field, the release profile of the dextran from the hydrogel subjected to an external magnetic field was
the same as the release profile of the dextran in samples stored in the absence of a magnetic field. This shows that the presence of a static magnetic field did not influence the release kinetics of our modelled therapeutic agent.

Figure 35: The effect of a static external magnetic field on the release profile of a 10 kDa dextran molecule.

However, if we factor in the results presented from the SQUID data presented above, we begin to understand that the results of this experiment are not surprising at all. From our SQUID experiments, it was observed that micro-particle size had a direct effect on the magnetic properties of our hydrogel system. Furthermore, from our results in our tangential force measurements in Aim 1, samples containing 500 nm NPs only produced a force of 1.70 mN. While this force may be large enough to activate the mechano-transduction pathways of encapsulated mesenchymal stem cells, it is far too low of a force to squeeze the rigid GMPs to accelerate the release profile of the dextran.
Cellularity:
Lastly, Human Fibroblast cells were encapsulated and incubated within our composite hydrogel scaffold for over 14 days. The data shown in Fig. 36 show that the addition of GMPs had no significant adverse effect on cellularity as expected, thus confirming the fact that cells are capable of surviving in our composite hydrogel system. It seems that in most of the samples, the cellularity within the scaffolds did not change very much across the different time periods. However, with scaffolds containing 120 μm micro-particles there was an increase in cellularity on day 4 when compared to the cellularity of the scaffold on day one. However, the cellularity decreases on to approximately the same values as day 1 on day 7.

Figure 36: Cellularity of Human Fibroblast Cells encapsulated in the hydrogel over 14 days.
4.4 Discussion

Scaffolds made for cartilage regeneration purposes must have sufficient porosity to allow adequate cellular migration and proficient diffusion of nutrients throughout the scaffold. Many synthetic biomaterials have been identified as potential candidates for cartilage repair, however, due to insufficient porosity, have failed to properly integrate with the host tissue\textsuperscript{141}. In this project, we have demonstrated a facile way to increase macro-porosity in a magneto-responsive biomaterial with potential applications for cartilage regeneration. By incorporating GMPs of different sizes into the hydrogel scaffold, one can easily control the degree of porosity within the hydrogel.

The data presented above demonstrated that it is possible to make a composite hydrogel scaffold that can be injected into the body, and over time, degrade to increase in porosity in the presence of collagenase. The addition of GMPs increased the hydrophilicity of the hydrogel thus having a statistically significant effect on the LCST and swelling behavior of the hydrogel at the time of formation. Unfortunately, it was also determined that the GMP size had a diminishing effect on the magnetic saturation of our magneto-responsive hydrogels. Thus, more work must be done to understand the optimum GMP size and loading needed to increase macro-porosity without having an adverse effect on the magnetic properties of our hydrogel.

However, despite the negative effect that the GMPs have on the magnetic properties of our hydrogel system, there are several other benefits to using GMPs in our hydrogel system. GMPs provide an attachment site for cells encapsulated within the hydrogel which is beneficial for long-term cell viability\textsuperscript{137}. Furthermore, GMPs have been used extensively for various drug delivery applications\textsuperscript{132}. Thus, for future experiments it would be
interesting to investigate the feasibility of encapsulating growth factors and other therapeutic agents that could be released as the GMPs degrade, thus promoting the formation of healthy cartilage tissue.

4.5 Conclusions

In conclusion, we would like to confirm that we were able to increase macro-porosity within our hydrogel by the simple addition of porogenic gelatin micro-particles. Micro-particles incorporation seemed to have no adverse effect on cellularity, gelation kinetics or hydrogel formation. However, the micro-particles did have a negative effect on the magnetic properties of our magneto-responsive biomaterials.

4.6 Recommendations/Future Directions:

Upon critical review of the data, there are a few recommendations that the author has to further the work presented above.

- **Quantification of Average Pore Size:** Due to time limitations, the author was not able to analyze the data presented in the SEM figure by quantifying the average pore size formed the degrading GMPs. We think it is important to complement our findings with from the SEM figures with some form of quantitative data to better understand the effect that the GMPs have on macro-porosity in our hydrogel system.

- **Cell Microscopy/Histological Staining:** Other potential future experiments that could be done to validate that the macro-porosity increases cellular infiltration within the hydrogel, is to employ histological staining techniques (Sanfrin’O, H&E,
Collagen or von Kossa staining) or advanced fluorescent microscopy to visualize how cells proliferate in our porous hydrogel scaffold.
Chapter 5: Effect of Short-Term Magnetic Stimulation on Stem Cellularity and Differentiation
(Research Aim 3)
5.1 Introduction

It has been well established in literature that mesenchymal stem cells commit to chondrogenic differentiation in response to extracellular mechanical forces.\textsuperscript{142} Chondrocytes exhibit compressibility and behavioral changes with compressive loads as a function of strain.\textsuperscript{106} The use of growth factors with loading has a collaborative effect on chondrocyte behavior. Mauck \textit{et al.} (2003) showed that chondrocyte stimulatory responses could be increased with the addition of TGF-\textbeta 1 and IGF.\textsuperscript{143}

Different strategies can be used to apply mechanical forces on cells, \textit{in vivo} and \textit{in vitro}. Recent studies have shown that mechanical cues, including the stiffness of a substrate, surface nanotopography, and extracellular forces, can direct stem cell fate \textit{in vitro} even with the absence of growth factors.\textsuperscript{85} Research has shown that, by mechanically stimulating the receptors on a stem cell surface, various mechano-transduction pathways such as Ras/MAPK, PI3K/Akt, RhoA/ROCK, and TGF-\textbeta pathways are activated causing the stem cells to commit to a chondrogenic lineage.\textsuperscript{104}

There are several techniques used in industry and academia to apply external forces on stem cells to initiate chondrogenesis. For example, the Flex Cell Tension system is a commonly used machine in industry that applies pressure on cells seeded on a silicone membrane that can stretch or flex in a certain direction.\textsuperscript{142} Studies have shown that cyclic and dynamic loading enhances protein biosynthesis while joint immobilization leads to degradation.\textsuperscript{144} Moreover, constant mechanical stimulation of cartilage cells is needed to improve their efficacy in joint repair. However, there have been very few innovative methods on how to stimulate the growth of cartilage cells through mechanical stimulation \textit{in vivo}. 
Therefore, the next grand challenge in reconstructing articular cartilage in vivo is trying to recreate an anisotropic tissue composed of superficial, middle and deep zones, where each zone varies in structure and function. From the data shown in Aims 1 and 2, we focused on the development of injectable scaffolds with spatiotemporally-controlled signaling to cause dynamic compressive and shear forces which will hopefully guide the regeneration of cartilage from heterogeneous and multi-tissue defects. In the data presented in this section, we present the results of some preliminary experiments used to evaluate cellular responses to locally induced shear and compressive stresses generated within the magneto-responsive hydrogel scaffold in response to alterations in magnetic field strength and frequency.

5.2 Experimental Methods

In Vitro Magnetic Field Stimulation

Harvested MSCs were added at a cell density of 1 million cells/mL to bionanocomposite hydrogels with 500 nm amine-functionalized NPs to obtain final concentrations of 313 and 625 μg of NPs/mL of hydrogel. The solutions were manually mixed, pipetted into 96 well plates, allowed to cross-link at 37 °C in an incubator for 1 hour, immersed with 2 mL media, and cultured for 7 days (changing the media every day). The hydrogels were subjected to an external magnetic field generated by an array of permanent magnets for intervals of either 30-40 min, 80-90 minutes or continuously on top of the magnet field for the whole duration of the experiment. For our negative control measurements, the bionanocomposite hydrogels were not stimulated by a magnetic field, and for the positive control, the hydrogels were cultured in osteogenic media with dexamethasone.
Hydrogels from each group were collected for analysis after 1, 4 and 7 days. 3 complete hydrogels were used for biochemical assays, and one hydrogel was used for fluorescent microscopy. Each of the hydrogels reserved for assays was submerged in 2 mL of PBS and then broken up using an 18 1/2 gauge needle repeatedly. Further homogenization was achieved by means of three consecutive freeze/thaw/sonication cycles (10 min at -80°C, 10 min at 37°C, 10 min sonication). As described in the literature, the viability of the MSCs encapsulated in the hydrogel was assessed by means of a Quant-iT Picogreen DNA assay (Molecular Probes, Eugene, OR). The values obtained from the DNA assay were normalized to the hydrogel weight. Inherent mineralization properties and the osteogenic differentiation of the encapsulated MSCs, mineralization was assessed by means of a Calcium assay. Alkaline phosphatase (ALP), an early marker of osteogenic differentiation, was measured using the Sigma-Aldrich ALP assay (Sigma, St. Louis, MO).

The bionanocomposite hydrogels were then removed and washed in PBS solution to remove the growth media. Samples were then collected and the DNA content in each hydrogel was determined with a Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific). ALP production was determined via an ALP Assay kit from ThermoFisher.

5.3 Results

In this preliminary study, the effect of short term magnetic stimulation and nanoparticle loading on MSCs behavior was investigated. Bionanocomposite hydrogels were created with 500 nm NPs at loadings of 313 and 625 μg/mL. Primary MSCs were encapsulated at a concentration of 1 million cells/mL of hydrogel, and the hydrogels were subjected to either a constant static field or cyclic stimulation of 30-40 min or 80-90 min with a 0.3 tesla magnet. After 7 days of stimulation, the hydrogels were collected, and DNA, calcium and
alkaline phosphatase (ALP) assays were conducted to determine the effect of magnetic field stimulation and nanoparticle loading on stem cellularity (Fig. 37), hydrogel mineralization (Fig. 38), and ALP production (Fig. 39), which is an early indicator for osteogenic tissue formation.

Figure 37: Effects of Short Term Magnetic Stimulation and NP loading on Stem Cell Viability
Figure 38: Effect of Short Term Magnetic Stimulation and NP loading on ALP Expression.
To better understand the effect that magnetic stimulation time and NP loading on stem cell viability and differentiation, a factorial analysis of the data above was performed to provide additional insight on how the encapsulated stem cells responded to the experimental parameters (Fig. 40). It was found that magnetic stimulation increases cellularity and hydrogel calcification increased with increases in NP loading but decreased with increases
in magnetic stimulation. Finally, ALP expression increased with NP loading, but decreased with magnetic stimulation. However, more experimentation is required to understand fully what is happening to the encapsulated stem cells in the bionanocomposite hydrogels when it is subjected an external magnetic field.

Figure 40: A full factorial analysis of the effect of nanoparticle loading and magnetic field stimulation on cellularity quantified by DNA assays (a), calcium mineralization within the hydrogel (b), and alkaline phosphatase (ALP) expression (c).

The data shown above suggests that the encapsulated MSCs in our magneto-responsive biomaterial are responsive to forces generated within the hydrogel structure when subjected to an external magnetic field. Next, we ventured to understand the different magnetic stimulation patterns influence stem cell behavior. For example, do cells respond favorably to patterned magnetic stimulation or static magnetic stimulation. To address this issue an
Arduino-automated magnetic field generator device was created to create a patterned magnetic field (Fig. 41). The effects of the different magnetic field stimulation patterns on ALP expression and hydrogel calcification are show in Fig. 42.

Figure 41: Experimental setup for an Arduino-controlled magnetic field generator. 6 electromagnets were connected to a microcontroller to create a patterned magnetic field.
Figure 42: Effect of patterned vs. static field stimulation on ALP expression and total hydrogel calcium content. Patterned magnetic field (6 hours – 30s on and 30s off, 0.03T), Static Field (6 hours, 0.3T)

From the results presented above, we did not observe appreciable differences between samples under patterned or static magnetic stimulation. One plausible reason for this may
be attributed to the fact that magnetic field generated by the electromagnets was determined to be about 0.03 tesla, which may be too low to see any statistically significant differences in stem cell differentiation. However, further experimentation must be done to fully comprehend the effect of static vs. patterned magnetic field stimulation patterns on stem cell differentiation.

5.4 Conclusions

The effect of short term magnetic stimulation on encapsulated stem cell behavior was determined. Through our factorial analysis, it was found that magnetic stimulation increases cellularity and hydrogel calcification increased with increases in NP loading but decreased with increases in magnetic stimulation. Moreover, ALP expression increased with NP loading, but decreased with magnetic stimulation.

5.5 Future directions

To be able to observe appreciable differences in stem cell differentiation caused by patterned or static magnetic fields, a large rare earth magnet was purchased (magnetic field strength of ~ 1 tesla) and was mounted on top of a linear actuator (Fig. 43). The new linear actuator setup can be controlled by a microcontroller, allowing the user to have more control on magnet field strength, and magnetic field stimulation time and frequency. We believe that with this apparatus, the effect of patterned vs. static stimulation on stem cell behavior would be more visible and easier to understand.
Figure 43: New experimental setup to study the effect of patterned vs, static magnetic field stimulation.
Chapter 6: Cisplatin loaded Injectable, Thermo-sensitive Hydrogel Scaffolds for the treatment of Ovarian Cancer
6.1 Introduction

During my tenure at Northeastern, I have had the wonderful opportunity to collaborate on a diverse portfolio of cross-functional, multi-disciplinary research teams to develop advanced biomaterials, drug delivery systems and nanoscale biosensors. For this section of this thesis, I wanted to present some results obtained from a side project done in collaboration Dr. Rajiv Kumar, Bryce Delgado, and Muizz Zaman from the Sridhar Lab at Northeastern University where we investigated the possibility of using an injectable thermo-responsive hydrogel system for the localized delivery of cisplatin (a chemotherapeutic drug) for the treatment of cervical cancer.

Cervical cancer affects over 500,000 women globally and is responsible for 280,000 deaths every year. Currently, there are several US Food and Drug Administration (FDA) approved drugs that could be used to treat cervical cancer. However, it remains an ongoing challenge to deliver these drugs to the tumor site as chemotherapeutic drugs administered either orally or parenterally are quickly metabolized in the body before reaching the tumor. Moreover, drugs delivered intravaginally have to pass through a protective mucus layer prohibiting the affected tissue from receiving the full dose of the drug. Therefore, the goal of this project was to develop an injectable hydrogel system that could be used to provide a sustained release of cisplatin to treat cervical cancer cells.

For several years scientists have sought to develop novel drug delivery platforms capable of delivering therapeutic agents to a target site in the body to treat various clinical disorders. Polymeric controlled drug delivery systems have become a subject of interest in this specific area of research as they have the potential to revolutionize the current conventional methods of drug administration in patients suffering from life-long illnesses.
such as cancer, diabetes and rhythmic heart disorders. Moreover, injectable polymeric biomaterials are particularly advantageous for long-term drug delivery applications because they are minimally invasive and avoid the complex surgical procedures associated with implantable drug delivery devices, thus reducing the need for regularly scheduled hospital visits, and the use of indwelling percutaneous lines needed to operate the implanted device.

Injectable stimuli-responsive biomaterials have emerged as ideal candidates for advanced self-regulated drug delivery systems. Stimuli-responsive biomaterials, capable of spatiotemporally moderating the release of a drug and/or therapeutic agents in response to physiological changes in their local environment, could be an extremely effective method to treat disease states that require rhythmic dosages delivered to the damaged tissue over a specific time interval. Furthermore, recent published articles have presented compelling in vivo studies that advocate for the use of biomaterials susceptible to changes in the pH, temperature, light, sound and magnetic fields as controlled drug delivery systems. The results of our experiments findings are summarized below.

6.2 Results
Hydrogel Formation
An injectable, thermo-sensitive hydrogel scaffold was developed for the controlled release of cisplatin at a target tumor site. The hydrogel was formulated by suspending cisplatin in a hydrogel consisted of a thermogelling macromer (PNIPAAm) crosslinked with a degradable PAMAM chemical cross-linker (Fig. 44). Previous research articles have shown that by adjusting the TGM: PAMAM ratio, one can effectively tune hydrogel swelling properties, thus controlling drug retention time in the hydrogel scaffold. Cisplatin was loaded into the hydrogel at a final concentration of 0.8 mg/mL.
Figure 44: Schematic showing the formation of a cisplatin-loaded injectable hydrogel for the treatment of cervical cancer.

**LCST Studies**

To evaluate the effect of adding cisplatin on the LCST of the thermosensitive hydrogel, DSC was used to measure the thermal transition temperature of the hydrogels post-formation (Fig. 45). From the data presented in the graph below, it appears that samples containing cisplatin tend to have a higher LCST, than samples without cisplatin. Furthermore, as the TGM: PAMAM ratio is increased, the LCST also increases due to the increased hydrophilicity within the hydrogel by the addition of the PAMAM macromer. However, most importantly, in all experimental conditions the LCST across all test groups is still well below body temperature thereby confirming that these composite hydrogel scaffolds could be used as injectable scaffolds *in vivo.*
Figure 45: Effect of Cisplatin and PAMAM concentration on the LCST of our hydrogel scaffold.

**In Vitro Release Studies**

Figure 46: ICP-MS provides release profile of platinum-based cisplatin from hydrogel in a liquid environment

Cisplatin release was monitored using inductively coupled Plasma-Mass Spectrometry (Fig. 46). In this experiment, hydrogels with the highest TGM: PAMAM ratio were used to determine the release profile of cisplatin from our hydrogel scaffolds. From the data
presented in this graph, we noticed that 70% of the cisplatin was released within 2 hours post-injection time. This rapid release suggests that the drug is being expelled too quickly from the hydrogel environment, thus to counteract this from happening, one may increase the PAMAM content in the hydrogel scaffold. However, the tradeoff to this action is that the increased PAMAM content may increase the LCST of the hydrogel above body temperature.

**Cell Viability Studies**

![Percent Cell Viability Post Hydrogel Cisplatin Exposure](image)

Figure 47: Developing an IC50 curve for the HeLa cell line provides an effective Inhibitory Concentration value to use when designing in-vitro experiments. MTS results demonstrate the hydrogel does not inhibit or deactivate Cisplatin as it exits the polymer scaffolds and enters media surrounding HeLa cells in vitro.

Lastly, to evaluate the effectiveness of using these injectable, thermo-sensitive hydrogels as drug delivery systems for cancer treatments, HeLa cells were exposed to the cisplatin loaded hydrogel scaffolds (Fig. 48). The results of our experiment show that the cisplatin released from the hydrogels decreased cell viability by almost 80% thus proposing that this injectable cisplatin loaded hydrogel formulation could be effective in killing cancerous cells at the tumor site. However, more experimentation has to be conducted *in vivo* to better
understand see the effectiveness of this hydrogel platform in treating cancerous cells in the body.

6.3 Conclusions

In conclusion, in this project, an injectable thermo-sensitive hydrogel that has potential to be used for the localized delivery of cisplatin to a target tumor site. The addition of cisplatin did increase the LCST of the hydrogel, however, the LCST was still well below body temperature, making the hydrogels suitable for injection into the body. Our drug release studies show that 70% of the cisplatin was released within the first two hours of our experiment, however, our cell viability tests confirm that, with the rapid release of the cisplatin, 80% of the HeLa cells that were exposed to the cisplatin loaded hydrogels were affected. Moving forward, further investigation on the efficacy of using these cisplatin-doped hydrogels in reducing tumor volume while minimizing toxicity associated with chemotherapy treatments will be evaluated in a cervical cancer tumor mouse model.
Chapter 7: Concluding Remarks
The primary objective of this research was to develop an injectable scaffold capable of responding to an external stimulus, such as a magnetic field, for cartilage regeneration application. Most of the current treatment options available do not regenerate or restore functional articular cartilage, thus it has become imperative to find an alternative solution to this growing problem. Tissue engineers have tried to fabricate scaffolds with strong mechanical properties and similar structural heterogeneity to that of articular cartilage by developing implantable scaffolds via 3D bioprinting, electrospinning, and creating multi-layered structures. However, the challenges with using implantable scaffolds is that they require invasive open surgery, elicit negative immune responses (e.g. fibrous capsules), and tissue defects of complex geometries can be problematic.

The challenges associated with implantable scaffolds thus gave rise to the need for the use of injectable scaffolds for cartilage repair. Many naturally derived and synthetic biomaterials have been used as injectable scaffolds, however, they often lack the mechanical integrity needed to repair cartilage tissue. Synthetic biomaterials are a preferred choice for injectable biomaterials as physical properties and degradation kinetics can be easily tuned with polymer chemistry. The next grand challenge in tissue engineering is to develop synthetic biomaterials capable of responding to an external cue to control/guide the regeneration of a heterogeneous tissue structure in vivo.

Nanocomposite hydrogels that can respond to a magnetic stimulus can be created by incorporating magnetic nanomaterials into a hydrogel network. Magneto-responsive hydrogels could be used as injectable scaffolds that can spatiotemporally manipulate cell behavior by generating local micro-shear and compressive stresses on encapsulated MSCs, thus activating cellular mechano-transduction pathways that could lead to the
differentiation of MSCs to chondrocytes. These magnetic systems could innovate and provide a viable solution to the unmet medical need of restoration of cartilage tissue damaged by OA.

Our research revolves around the development of a bionanocomposite hydrogel was made by using a thermogelling macromer, p(NiPAAm\textsubscript{92.5}-co-GMA\textsubscript{7.5}), to give pendant epoxy rings in the polymer backbone. \textsuperscript{1}H NMR, GPC and SEM were used to confirm polymer composition and nanoparticle incorporation into the hydrogel scaffold. Furthermore, a mathematical model was developed to predict potential forces that could be generated within the hydrogel and the forces predicted were approximately 3 times greater than those measured in the tangential measurements and SQUID experiments confirmed that NP loading and size have a significant effect on the hydrogel response to an external magnetic field.

Next, we investigated the feasibility of increasing macro-porosity within our hydrogel scaffold by adding degradable gelatin micro-particles into our hydrogel formulation. We found that micro-particles incorporation into our hydrogel seemed to have no adverse effect on cellularity, gelation kinetics or hydrogel formation. However, the micro-particles did have a negative effect on the magnetic properties of our magneto-responsive biomaterials by diminishing the magnetic properties of our magneto-responsive hydrogel.

Lastly, we investigated the effect of short term magnetic stimulation on encapsulated stem cell behavior. In our preliminary magnetic stimulation experiments, it was found that magnetic stimulation increases cellularity and hydrogel calcification increased with increases in NP loading but decreased with increases in magnetic stimulation. Moreover, ALP expression increased with NP loading, but decreased with magnetic stimulation.
References:

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Appendix
Figure 48: $^1$H NMR Spectra if p(NiPAAm92.5%-co-GMA7.5%) with proton peak.
Figure 49: $^1$H NMR Spectra of PAMAM with proton peak locations identified and an experimental average molecular weight of ~2100 Da was calculated.
Figure 50: Oscillatory rheology traces showing dual thermal and chemical gelation for injectable nanocomposite hydrogel solutions with DSPE-Amine functionalized MNPs (40 nm and 500 nm).