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Spatial Control of Magnetic Nanoparticles Integrated with Cellular Spheroids as Tissue Engineered Building Blocks

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A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Brandon Mattix
August 2013

Accepted by:
Dr. Frank Alexis, Committee Chair
Dr. Jiro Nagatomi
Dr. Dan Simionescu
Dr. Richard P. Visconti
Abstract

Magnetic nanoparticles (MNPs) have been investigated in tissue engineering applications to provide in situ imaging, drug delivery, and tissue patterning, but direct and prolonged interaction between cells and MNPs can have adverse effects on cell function. Therefore, methods which reduce or limit the interaction of MNPs with cells, or utilize more biocompatible MNP-based strategies will improve upon the commonly used iron oxide MNPs. We investigated a variety of methods to improve upon the use of MNPS in tissue engineering.

Cell aggregates, or spheroids, have been used as tissue engineered building blocks that can closely mimic the native three-dimensional in vivo environment. Current strategies incorporating MNPs into tissue engineering often involve cellular uptake, however, which can induce adverse effects on cell activity, viability, and phenotype, and should therefore be avoided. Here, we report a Janus structure of magnetic cellular spheroids with spatial control of MNPs to form two distinct domains: cells and extracellular MNPs. This separation of cells and MNPs within magnetic cellular spheroids had no adverse affects on long-term viability or cellular phenotype, allowing for the magnetic manipulation and fusion into controlled patterns and complex tissues.

Iron oxide NPs are the most common MNP in biomedical applications, but these MNPs often require complex surface modifications to improve their biocompatibility. We report the preparation of magnetoferritin NPs, a biological MNP, capable of serving as a biological alternative to iron oxide MNPs. Magnetoferritin NPs were incorporated into
Abstract (continued)

three-dimensional cellular spheroids with no adverse effects on cell viability and were capable of magnetic force assembly into fused tissues.

Additionally, the ideal nanomaterial will remain stable for a sufficient amount of time to accomplish its desired task, and then rapidly degrade once that task is completed. We report the use of surface modifications to accelerate iron oxide MNP degradation mediated by polymer encapsulation in polymers with different degradation rates: poly(lactide) (PLA) or copolymer poly(lactide-co-glycolide) (PLGA). Results demonstrated that the degradation of MNPs can be controlled by varying the content and composition of the polymeric nanoparticles used for MNP encapsulation (PolyMNPs). These PolyMNPs maintained a high viability compared to non-coated MNPs, and are also useful in magnetic force assembly into fused tissues. The presented results highlight multiple strategies which can improve upon the biocompatibility of MNPs in tissue engineering applications.
DEDICATION

I would like to dedicate this work to my family, friends, and anyone who has ever motivated me to continue pursuing my dream. Additionally, I would like to my Lord and Savior for blessing me with these opportunities and the amazing people I can call my family and friends.
ACKNOWLEDGEMENTS

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I would like to express my sincere gratitude to my graduate research advisor, Dr. Frank Alexis, for his support, patience, and supervision throughout my studies. His willingness to go above and beyond of what is expected, as well as his constant drive have pushed me to accomplish tasks I thought unattainable. Additionally, I would like to express my deep appreciation for my committee members’ input and guidance throughout my research.

I would like to thank Dr. Rick Visconti for hosting me at MUSC and for constant experimental support, as well as Jing Zhang, Agnes Mehesz, and Chris Fuchs for their support with IHC and flow cytometry. I want to thank Dr. Terri Bruce and Rhonda Powell of the Clemson Light Imaging Facility for help with microscopy and constant guidance; Linda Jenkins for her assistance with histology; Kim Ivey for her help with TGA analysis; and Dr. Roger Markwald for his guidance regarding challenges and innovations in tissue fabrication. Furthermore, I would like to thank George Fercana and James Chow as well as Dr. Simionescu and members of the BTRL lab for their help with IHC, histology, and equipment; Dr. Nagatomi and the members of the Mechanobiology lab, and Dr. Burg and members of IBIOE for their support and help with equipment.

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CHAPTER 1

BACKGROUND

SMALL DIAMETER VASCULAR GRAFTS

Each year, thousands of patients are unable to receive organ implants due to a shortage of available donor organs. The field of organ transplantation to replace or repair damaged tissues is dependent solely upon the number of available donor organs, with the number of waitlisted patients increasing annually [1]. The American Heart Association estimates that the total number of cardiovascular operations and procedures increased 28% from 2000 to 2010, with 7,588,000 procedures performed in 2010, ranking cardiovascular disease as the highest among all disease categories in hospital discharges [2]. For vascular engineering, the majority of vascular diseases and failures occur in blood vessels less than 6 mm in diameter [3]. Though autologous vascular tissue from the host is the most preferable solution when available, it is estimated that 30% of patients with vascular problems lack sufficient vasculature for autologous replacement due to abnormalities or damaged tissues [4]. The use of commercially employed grafts is currently limited to autografts, allografts, and xenografts, as well as synthetic ePTFE and Dacron. However, human clinical trials have been recently begun for engineered vascular grafts made from novel polymers [5] and cell sheet technologies [6]. As the forces experienced by the different types of vessels vary, a primary challenge in tissue engineering vascular grafts involves matching native mechanical properties. Additionally, the different vessel types contain diverse cellular and extracellular matrix (ECM) compositions that mediate their mechanical properties and function. Therefore, a
variety of approaches have been investigated for the assembly of a small diameter vascular graft capable of restoring native functionality that also integrates with the host to negate the need for eventual graft replacement. These strategies, along with their shortcomings and potential solutions, are detailed in the following sections.

1.1. Biological Vascular Grafts

1.1.1. Autograft

For small diameter blood vessel replacements, arterial and venous autografts are typically the optimum procedure used (Figure 1.1) [7]. The internal mammary artery (IMA) and radial artery are preferred for coronary artery bypass grafts [8]. However, for lower limb and peripheral bypass procedures, saphenous vein grafts (SVGs) are commonly used. The predictors of autograft patency include the size of recipient vessel (length and diameter) [9], the particular disease of the patient, and the methods used to preserve the grafts [10]. Though cold storage has been associated with long term graft patency, primarily attributed to its preservation of the inherent endothelial function, these grafts are still susceptible to common modes of failure such as atherosclerosis and intimal hyperplasia [11, 12]. Additionally, it is estimated that 30% of patients requiring vascular replacement lack sufficient vasculature for grafting due to damaged vessels [4], typically attributed to inadequate quality of vessels from either prior disease due to a lack of vasculature due to prior use [13].

The use of autografts over other grafts is primarily due to their high long-term patency rates. Goldman et al. compiled data from 1254 patients analyzing long-term results of IMA and SVGs for coronary artery bypass grafts [14]. Subsequent analysis
confirmed the differences in patency between the two autologous sources, with overall patency rates of 61% for SVGs and 85% for IMA grafts at 10 years. These results further confirm the difficulty associated with small diameter vascular replacement, as autografts still encounter difficulties.

1.1.2. Allograft

Though the high availability and easy storage of allografts (via cryopreservation) permit their quick use, difficulties arise due to inabilities to tailor matrix content and dimensions (Figure 1.1). Additionally, they have an inherent risk of viral transmission [15], are subject to breakdown from processing [16], and also exhibit poor long-term patency [17, 18].

Though various studies have been performed to analyze the effectiveness of cryopreserved commercial allografts, results have revealed contradicting conclusions based on sample size, methods of preservation, patient population, and use of pharmacological agents [19]. In their study of 240 cryopreserved allografts implanted in lower limbs using commercial CryoLife Cryografts over the course of 10 years, Farber et al. found these allografts to be characterized by poor primary patency, with rates of only 83% at 1 month, 50% at 6 months, 30% at 12 months, and 18% at 24 months [20]. Additionally, mortality rates were higher than that reported by other studies, in which only 72% patients remained alive after year one with a subsequent decrease to 69% at the end of year two. Causes of graft failure included thrombus formation, wound infection, seroma formation, and aneurysm [17, 19, 20]. Though results suggest that these grafts
may be sufficient to prevent immediate limb loss, they are ineffective as a long-term replacement strategy [19].

1.1.3. Xenograft

Vascular xenografts can come from tissues other than blood vessels, including the submucosa of the small intestine (Figure 1.1) [21, 22]. Although readily available, xenografts are not commonly used in vascular replacements due to immunorejection, low patency rates [23], and the need for additional treatments to reduce thrombotic responses [21]. One method for improving xenograft function entails in vivo endothelialization, in which the scaffold is implanted subdermally within the host and populated with autologous cells prior to desired use [24].

Commercially available xenografts for vascular replacements are also available. Clinical results have demonstrated the ArteGraft’s superiority as an AV access graft compared to ePTFE grafts, with primary patency rates at one year of 60.5% compared to 10.1% of synthetic grafts [25]. However, clinical trials comparing the ProCol (collagen cross-linked vascular solution derived from the bovine mesenteric vein) to ePTFE grafts for AV access grafts showed low patency (1 year primary patency of 36% and 28% for ProCol and ePTFE, respectively) [23].
Table: Grafts and Their Pros and Cons

<table>
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<th>Graft</th>
<th>Pros</th>
<th>Cons</th>
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| Autograft | • Biologically-based                                 | • Secondary surgical site                                  | ![Autograft Diagram](https://via.placeholder.com/150)
|           | • High long-term patency                             | • Inability to tailor dimensions                           | ![Autograft Diagram](https://via.placeholder.com/150)
|           | • Autologous source                                  | • Limited supply                                           | ![Autograft Diagram](https://via.placeholder.com/150)
| Allograft | • Readily available                                  | • Inability to tailor matrix contents                      | ![Allograft Diagram](https://via.placeholder.com/150)
|           | • Biologically-based                                 | • Risk of viral transmission                              | ![Allograft Diagram](https://via.placeholder.com/150)
|           | • Long shelf-life                                    | • Mechanical breakdown from processing                     | ![Allograft Diagram](https://via.placeholder.com/150)
| Xenograft | • Readily available                                  | • Risk of immunorejection                                  | ![Xenograft Diagram](https://via.placeholder.com/150)
|           | • Biologically-based                                 | • Require graft modifications                              | ![Xenograft Diagram](https://via.placeholder.com/150)
|           | • Long shelf-life                                    | • Poor long-term patency                                   | ![Xenograft Diagram](https://via.placeholder.com/150)

Figure 1.1. Biological Vascular Grafts. Grafts can be harvested from a variety of sources, including the patient (autograft), another person (allograft), or an animal (xenograft). Though autografts are currently the best method for small diameter vascular grafts, patients may lack sufficient vasculature to harvest, in which case other grafts may
be used [26]. Allografts and xenografts both require treatment to decellularize the vessels prior to use, which introduces problems such as mechanical breakdown and the inability to tailor vessel dimensions or composition [27, 28].

1.2. Polymeric Engineered Vascular Grafts

1.2.1. ePTFE and Dacron

The limited availability of autologous conduits has led to a widespread use of synthetic grafts. The main materials for engineered synthetic grafts include woven polyethylene terephthalate (PET), more commonly known as Dacron, and expanded polytetrafluoroethylene (ePTFE). The use of either ePTFE or Dacron has shown no significant differences regarding patency between each in small diameter graft applications, with three-year patency rates of approximately 60% for both [29, 30].

Although these grafts have been used successfully in large diameter vascular grafts, low long-term patency rates render them ineffective in small diameter settings, a phenomenon that is often attributed to compliance and mechanical mismatch [7-9]. The primary deficiencies of these materials include an increased rigidity compared to native vasculature [10] and a lack of endothelial lining (Figure 1.2) [11-16]. In small diameter applications, this can cause thrombus formation often attributed to compliance mismatch, which in turn results in graft failure [10, 17, 18]. Research has been performed to improve small diameter synthetic grafts via functionalization to promote cell adhesion using a variety of biomolecules, including fibronectin [19, 22], collagen [22], cell adhesion peptide sequences [21, 23, 24], and growth factors [25, 31-33]. Results have shown improved cell adhesion compared to bare grafts, thereby potentially improving graft functionality. The risk factors inherent in using these small diameter ePTFE and
Dacron grafts include age, gender, smoking, hypertension, and graft diameter [29]. Typically used in large diameter applications, these grafts have a low patency in small diameter applications below the knee [7-9], with larger diameter grafts (7-8 mm diameter) demonstrating higher three-year patency rates than their smaller (5-6 mm diameter) counterparts.

1.2.2. Biodegradable Polymeric Scaffold

A variety of polymers have been analyzed for use in the fabrication of engineered vascular grafts due to their high availability, low cost, and ability to be fabricated into controlled shapes and dimensions. Biodegradable scaffolds have been increasingly used as vascular grafts that will initially provide mechanical support while promoting tissue infiltration and growth, eventually being replaced entirely by native tissue (Figure 1.2). Common biodegradable polymers investigated for use in vascular engineering include polyglycolic acid (PGA) [34], polyurethanes (PUs) [35], polycaprolactone (PCL) [5], and polylactic acid (PLA) [36-39]. One of the most often used methods for the fabrication of biodegradable polymeric vascular grafts is electrospinning [40-44]. This scalable and easily customized method allows for control of electrospun fiber diameter and orientation, scaffold porosity, spatial location of heterogeneous graft components, and graft material properties based on fabrication parameters. Compared to other nanofiber fabrication techniques (e.g. drawing, template synthesis, temperature-induced phase separation, and molecular self-assembly), electrospinning is advantageous because its results are reproducible, scalable, continuous, and can be easily tailored to assemble either micro-or nano fibers using the same experimental set-up with a variety of biologic
and synthetic polymers [40, 45]. By controlling the nanofiber deposition, it is possible to create electrospun vascular grafts that closely mimic the native extracellular matrix scaffold within vessels, while also mediating cellular interactions and orientation within electrospun grafts based upon such criteria as the diameter [46], porosity [47], and surface topography of a given fiber [48]. Synthesis parameters, such as polymer concentration (viscosity), jet radius, flow rate, electrical potential, solution conductivity, and distance between nozzle and collector mediate fiber diameter and morphology and can be tailored based on desired properties [45, 49, 50]. Altering these parameters enables the creation of fiber diameters ranging in size from 50 nm up to 12 µm [51, 52]. Additionally, the porosity of electrospun scaffolds can be tailored to promote cell infiltration, with pore sizes ranging in size from 2-465 µm and porosities up to 92% [53, 54]. Furthermore, the ability to fabricate multilayered scaffolds can be used to improve graft biocompatibility and mechanical properties. In their efforts to assemble an electrospun bilayered scaffold with an inner layer of elastin, Wise et al. mimicked native vascular structures by utilizing elastin and PCL. Elastin improved the biocompatibility and interaction with blood and circulating cells. The inner elastin layer was surrounded by an external layer of PCL, which provided scaffolds with mechanical support [55]. Other groups have demonstrated spatial control over fiber diameter and porosity, successfully assembling grafts containing highly porous inner layers, which support cell adhesion and infiltration, within the lumen and more dense external layers to provide mechanical support [56, 57]. The ability to assemble tailored scaffolds that can closely mimic the structure and scale of native ECM makes electrospun scaffolds a good
candidate for small diameter vascular grafts. However, a strong understanding of how scaffold properties (fiber diameter, porosity, copolymer orientation, fiber alignment) mediate its interaction with the local biological environment and eventual biodegradation must be established to optimize scaffold properties.

Polyglycolic acid (PGA) is one of the most commonly investigated polymers for use in creating degradable vascular scaffolds due to its rapid degradation compared to other polymers [34, 58]. As one of the fastest degrading polymers in biological environments, PGA has shown limitations in maintaining long-term mechanical properties, with studies demonstrating a complete loss of tensile strength in raw PGA scaffolds after a three week incubation period in an aqueous environment [59]. However, this loss of scaffold support is balanced by the additional strength of cell penetration and proliferation, as well as ECM generation and secretion. Results have shown that samples seeded with cells mechanically improve with culture time, demonstrating an almost four-fold increase in burst strengths between five and eight weeks in culture [59]. Another interesting characteristic of PGA scaffolds is the in vitro dedifferentiation of smooth muscle cells (SMCs) from PGA degradation byproducts [34, 59-61]. This dedifferentiation of SMCs raises concerns for vascular failure, as intimal hyperplasia has been correlated to the proliferation, dedifferentiation, and migration of SMCs into the intima of vessels [62-64]. While biodegradable PGA vascular grafts have shown some success in vivo, their rapid degradation and adverse effects related to degradation byproducts hinder their use in small diameter vascular grafts.
Polyurethanes (PUs), another common biodegradable polymer, have also been investigated for use as a vasculature material. Though the viscoelastic compliant nature of PUs is a suitable alternative polymeric vascular material for improving upon the low compliance of other polymers, clinical results have been mixed, chiefly due to degradation mechanisms and byproducts [35, 65]. Though PUs exhibited positive tissue in-growth as well as reduced anastomotic hyperplasia in clinical trials [66, 67], significant rates of thrombosis and infection [68], and variable rates of patency [67-70] have also occurred with their use. Additionally, while poly(ether)urethanes (PEtU) polymers have been used in medicine for 40 years, the thrombogenecity and aneurysm formation within these polymers in various vascular solutions have resulted in clinical outcomes that have been quite varied [65, 68, 71]. In their research to construct an engineered vascular graft 5 mm in diameter, Soldani et al. used a PEtU macromolecule containing poly(dimethyl siloxane) (PDMS), also known as silicone [35]. Porous biodegradable PEtU grafts were implanted into an adult sheep carotid artery model and compared to ePTFE grafts up to 24 months. The grafts contained two distinct regions, a highly porous internal layer and a low porosity external layer. The use of a porous lumen surface was also chosen based on previous studies that showed high porous luminal surfaces are less thrombogenic than non-porous surfaces [72]. PEtU grafts maintained patency up to 24 months, with grafts almost completely biodegraded and replaced by fibro-connective tissue. A noticeable radial enlargement was observed, however, a potential sign of future graft failure via aneurysm. Additionally, the potential adverse
effects related to PEtU degradation byproducts may limit their clinical applications [73, 74].

Polymer selection is a critical for vascular engineering in that the mechanical properties and the degradation kinetics of scaffolds can be tailored based upon polymer properties. A fundamental property of polymeric vascular grafts is compliance and elasticity, with polymers exhibiting a wide range of elastic modulus based on the polymer selected. These polymers exhibit elastic modulus ranging from elastic PCL (4.98 MPa) [75] to more rigid PLLA (56 MPa) [76]. Polymer mechanical properties are important in that graft stiffness plays a key role in overall success, with differences in elastic modulus leading to the formation of intimal hyperplasia [17].

Using a biodegradable copolymer, clinical trials have recently begun for the first ever tissue-engineered blood vessel in the United States [77]. A copolymer of L-lactide and ε-caprolactone (50:50) was used to fabricate a polymeric scaffold supported with a PGA woven fabric. Grafts exhibited approximately 80% porosity, with pore sizes from 20 to 100 µm, to allow for autologous bone marrow cell seeding and penetration prior to implantation [58]. Although the implanted grafts cannot be classified as small diameter (12-24 mm diameter), these results are still promising for use of polymeric scaffolds in all vascular applications. In the median follow-up analysis of 5.8 years, no evidence of aneurysm formation, graft rupture, graft infection, or calcification was present [5]. While these results are promising for vascular grafts, the investigation into small diameter applications using this strategy must be examined.
While clinical trials are now underway on the first biodegradable polymer vascular graft for large diameter applications, clinical trials for small diameter biodegradable vascular grafts have yet to be undertaken. Data for biodegradable vascular grafts in small diameter applications is often limited to short-term *in vivo* results (less than six months), with potential problems often arising after this six month period. Groups that have analyzed more long-term studies over six months have highlighted some causes for concern regarding biodegradable vascular grafts. One group analyzed 2 mm electrospun PCL vascular grafts at various time points up to 18 months in rats to determine their long-term fate [78]. Results showed that scaffolds maintained patency and mechanical properties while allowing efficient endothelialization, but insufficient regeneration was seen in the vascular wall over time. Endothelialization, cell invasion, and neovascularization increased over a period of six months, but regressed at 12 and 18 months, leading to eventual calcification of grafts. Identical results have also been observed in collagen based vascular grafts after six months in a canine model, with evaluation performed up to 12 months [79]. In addition to this cellular regression, biodegradable vascular grafts also have deficiencies related to degradation kinetics. Specifically, the degradation rate of these grafts, as either mechanical failure or variations in graft degradation kinetics, can lead to unpredictable graft failures. Overall, though biodegradable vascular grafts have numerous advantages related to fabrication of controlled structures, complications related to cellular regression and degradation kinetics and byproducts must be elucidated and understood.
**Figure 1.2. Polymeric Vascular Grafts.** Polymeric grafts can be separated into two categories: permanent and biodegradable. Permanent polymeric grafts are made of ePTFE and Dacron, and serve well in large diameter applications [29]. However, they encounter problems in small diameter vessels, often attributed to mechanical mismatch [10]. Biodegradable polymers serve initially as a mechanical support, but eventually degrade and are replaced with native tissue. Difficulties often arise when attempting to tune degradation kinetics, mechanical properties, and balancing degradation products [35, 80].
1.2.3. Natural Polymers

Natural biopolymers such as collagen [81, 82], fibrin [83, 84], elastin [85], chitosan [86, 87], and silk [88, 89] have also been investigated for use as vascular grafts. Though the biological origin of these materials provide natural cell attachment sites and improved tissue integration compared to synthetic polymers, purification and isolation of these materials often makes them mechanically weak and expensive to produce [90]. These grafts can, however, be chemically modified or mixed with synthetic polymers to improve their mechanical properties [55, 82, 91]. For example, Lee et al. evaluated electrospun vascular grafts composed of collagen, elastin, and various polymers including PLGA, PLA, PCL, and PLCL [92]. Results confirmed that mechanical properties could be tailored via control over the synthetic polymer used, with the elastic modulus increasing between pure collagen and elastin grafts (0.44 MPa) and grafts containing PLGA (0.85 MPa) or PLA (2.08 MPa). Primarily due to their poor mechanical properties when used alone, natural biopolymers function best in composite materials, in that they reduce thrombogenicity, improve biocompatibility, and improve tissue integration compared to pure synthetic polymers often used.

1.3. Scaffold-Free Vascular Grafts

Challenges related to polymeric vascular grafts have led to the development of engineering strategies that allow cells to self-assemble and form vascular constructs without the necessity of an architectural scaffold. Utilizing cell adhesion, self-assembly, and the cell’s inherent ability to produce ECM, scaffold-free approaches provide a
promising approach for the fabrication of small diameter vascular grafts. The primary drawback of this technique, however, is the requirement for large quantities of autologous cells, a costly and time-consuming endeavor [93]. However, various techniques have shown great promise, with some already in human clinical trials [6]. An overview of scaffold-free vascular grafts is provided in Figure 1.3.

1.3.1. Cell-Sheet Based Strategies

Cell-sheet technologies utilize autologous cells grown in vitro to synthesize a sheet of living tissue that can be used to assemble a more complex tissue. Cells can be grown on either thermoresponsive polymer films [94] or in special growth media conditions [95] that stimulate ECM secretion, thereby causing the cells to create a solid tissue sheet capable of manipulation. These sheets can then be layered sequentially to control spatial cell location of multiple cell types within a tissue while maintaining both cell interactions and ECM. These techniques have been applied to assemble myocardial [96, 97], corneal [98, 99], oesophageal [100], and even dental [101] tissues which have shown success in animal models.

Much research has been undertaken to develop cell-sheet based strategies for the creation of scaffold-free vascular grafts [95, 102]. Specifically, Cytograft Tissue Engineering recently published the results from Phase I & II clinical trials performed using their patented Lifeline autologous cell-based vascular grafts. L’Heureux and colleagues developed a tissue engineered blood vessel from autologous cells with mechanical properties comparable to native saphenous veins [95]. In 2009, nine patients received Lifeline grafts as arteriovenous (AV) shunts for haemodialysis access, in which
autologous fibroblasts and endothelial cells were obtained via biopsies and harvested in vitro [6]. Overall, primary patency was maintained in 78% of the patients at one-month post-implantation, and in 63% after six months. Though the results of this study demonstrated a potential fabrication technique for a complete biological and autologous vascular graft, the long fabrication times (6-9 months) and high autologous cell quantities required to produce such grafts currently mitigates their widespread use. Reducing both the fabrication times and cell quantities must be accomplished prior to making such grafts commercially viable [93].

Another company, Humacyte, is currently investigating the use of allogenic SMCs to create a cell-derived acellular scaffold. Allogenic cells are seeded onto a PGA scaffold and physically stimulated for two months to accelerate ECM secretion and induce maturation using cyclic radial strain, followed by decellularization, which results in a collagenous matrix. Results demonstrated high patency and a resistance to dilation, calcification, and intimal hyperplasia in a baboon model as AV conduits [80], indicating their viability as vascular grafts in cell-derived acellular scaffolds.

1.3.2. Aggregated Cell Constructs

Similar to cell-sheet based methods, this technique does not involve decellularization that results in an ECM-based scaffold, but does utilize cellular self-assembly as a vascular graft. Using a circular agarose mold, Gwyther et al. successfully fabricated SMC-based rings with inner diameters of 2, 4, and 6 mm [103]. Results showed that individual rings, as well as stacked rings to form tubes, could be fabricated in one to two weeks in static culture [104]. Mechanical testing to quantify ultimate tensile
strength (UTS), stiffness, and toughness decreased (40% decrease in UTS in 6 mm rings) for each sample as culture time increased due to necrosis in the core of the ring. Additionally, mechanical properties of aggregated SMC rings were still much lower than native arteries [105].

Other groups have utilized cellular spheroids to assemble vascular tissues. Kelm et al. assembled human artery-derived fibroblasts and human umbilical cord vein endothelial cells to form three-dimensional cellular spheroids [106]. Spheroids are appealing for the assembly of complex tissues due to their ability to secrete ECM. Results comparing 3D spheroids to 2D monolayers revealed a dramatic increase in the expression of matrix-related gene transcripts such as collagen, fibronectin, laminin, glycosaminoglycans (GAGs), and matrix metallopeptidases (MMPs). Thousands of individual spheroids were seeded in a custom-built bioreactor and subjected to both dynamic flow and static conditions. Using 4,000-5,000 spheroids, a 5 mm long tube was fabricated with a wall thickness of 1 mm in only 14 days. Although not yet proven in vivo, strategies that utilize cells alone present a novel approach to vascular fabrication. By allowing cells to self-assemble and secrete their own ECM, tissues can dynamically adapt to meet required conditions.

Overall, scaffold-free vascular grafts have great potential in vascular engineering due to their purely biologic nature. By allowing cells to self-assemble into required densities and orientations, these vascular grafts can dynamically remodel to meet their required needs. Additionally, these grafts can secrete their own ECM, providing further mechanical support and dynamic remodeling. Challenges still exist regarding clinical
applications, however. The harvesting, growth, and eventual assembly of engineered grafts from autologous cells is both costly and time consuming, requiring months for the complete fabrication of a functional vascular graft, which in turn prevents any scale-up of this process. Furthermore, while some techniques have demonstrated comparable mechanical properties [107], most vascular grafts assembled using scaffold-free approach cannot match the mechanical properties of native vasculature, increasing the risk of graft failure due to mechanical mismatch. Consequently, elucidating the factors accelerating graft assembly and maturation is imperative for optimizing these fabrication techniques.

1.3.3. Tissue Printing and Patterning

Attempts to simulate the orientation, density, and types of cells found within native tissues have been thoroughly investigated as means to build engineered organs and tissues via precise control and deposition of cells and ECM components [108]. In embryonic development, specifically in a process known as morphogenesis, organs and tissues self-assemble and align into complex networks of cells and ECMs to form a particular tissue type [109]. Therefore, attempts to mimic this natural self-assembly offer a promising approach in tissue engineering. Various techniques have been investigated for cell and organ printing, including inkjet printing as well as “Bioink” deposition onto “Biopaper”. These techniques can be viewed as an automated, computer-aided, rapid prototyping technology capable of 3D tissue fabrication via individual cell or cell aggregate deposition into controlled patterns. Conventional cell-seeding techniques involve a suspension of cells within a solution and subsequent incubation on either a scaffold or surface. This offers limited to no control of seeding density or spatial
alignment, however. Additionally, these techniques are limited to monolayer deposition, which is characterized by poor tissue infiltration. Therefore, additional approaches for cell printing have been created that offer more precise control over conventional cell suspension techniques, which also show much promise for the tissue printing of engineered blood vessels (Figure 1.3) [110-112].

Inkjet printing has been used in many endeavors involving the printing of biomolecules into desired patterns and orientations [113], including growth factors [114, 115], DNA [116, 117], proteins [118, 119], cells [120], and bacteria [121] using modified inkjet technologies. Although mammalian cells are temperature-sensitive (250-350 °C), the small time scale of heating (µs) is insufficient to significantly affect the surrounding ink, therefore avoiding adverse effects on cell viability [120]. Analysis of the viability of printed cells using inkjet printers vary, ranging from 50% up to 98% depending upon the system configuration (thermal, piezoelectric, pressure-driven) and printing parameters [119, 120, 122-124]. Reduced viability from inkjet printing is primarily due to either shear stresses experienced during deposition or dehydration from droplet evaporation [122].

The use of a cellular inkjet device is one such potential method for the rapid fabrication of tissues [125]. Although 2D printing is now considered as an established concept, sequential 3D cell printing remains the ultimate goal for truly comprehensive tissue engineering. However, this technique has been demonstrated by multiple groups using both cellular [126, 127] and acellular [128, 129] substrates. Cellular aggregates have been successfully deposited on sequential layers of collagen and other
thermoreponsive gels, with aggregates demonstrating successful fusion between gel layers [126, 130]. Furthermore, acellular 3D alginate tubes have been printed using inkjet technologies that are capable of withstanding physiological fluid flow through its lumen [129].

Compared to inkjet strategies with cells in suspension, other strategies can print bioink aggregates composed of cellular and acellular substrates. This bioink composition can be controlled and optimized for desired tissue types, allowing for the “ink” to be deposited in a maximum cell density, while also reducing cell damage related to other printing strategies. Biopaper is a biocompatible gel that serves as a supporting environment to hold the bioink aggregates in place to allow for fusion and formation of the multicellular tissue. Collagen [126, 131] and agarose [132] have already been established as excellent supports (biopaper) for bioink fusion and aggregation. This particular cellular printing technique, via printing and fusion of bioink aggregates, has been successfully used to fabricate a scaffold-free tubular construct [132]. Here, the biopaper is not an actual “sheet” of material, but rather an extruded cylinder of the material itself. Ink aggregates were formed via suspension of cells in a capillary micropipette, creating a slurry mix capable of manipulation and handing. Cylindrical bioink rods were then mechanically extruded using a custom bioprinter. To form spherical particles, these rods were mechanically cut into small segments which demonstrated self-rounding capabilities. Agarose rods were also mechanically extruded and used as supporting biopaper. Though collagen was initially used as the biopaper, an uneven gelation in layer-by-layer deposition resulted in a noticeably imprecise product.
Additionally, collagen incorporation within the tube during fusion made removing the biopaper template difficult, so agarose was instead used. Agarose rods (biopaper) and spheroid aggregates (bioink) were carefully deposited in a variety of patterns in a layer-by-layer fashion to form 3D structures. Samples were allowed to fuse for 5-7 days prior to examination. Results demonstrated that tubes with diameters ranging from 900 µm up to 2.5 mm could be successfully fabricated, as well as branched networks with varying diameters. Furthermore, tubes could be patterned with different cell types to enable both self-assembly and the formation of heterocellular constructs, which in turn permitted the precise control over construct size, thickness, and pattern. Though these results have demonstrated that formation of vascular grafts is possible, both mechanical properties and in vivo studies have yet to be reported. Although promising, the use of spheroids can prove difficult as fabrication techniques are problematic to upscale. Each vessel formed requires a significantly large number of spheroids. For example, approximately 4,000 spheroids (300 µm diameter) were necessary to form a tube 10 cm long with a diameter of 1.5 mm [132]. In that both the fusion and maturation of grafts is a time consuming process, additional maturation via bioreactors and other techniques will likely be necessary after initial fusion and formation. Finally, removal of the agarose rod from the lumen of the vessel is done via manual pulling of the rod, therefore limiting its use in branched network formation as well as potentially damaging cells along the lumen.

Xu et al. recently printed a 3D zigzag cellular tube using a custom bioprinting system. Fibroblasts were suspended in sodium alginate to serve as a bioink that allowed for precise spatial placement of bioink beads (in X, Y, and Z directions) that maintained
cell viability post-printing. The overhanging section of the printed tube collapsed, rendering it unusable, however. Although these results failed to improve upon the mechanical challenges encountered in tissue printing, they demonstrate that bioprinting systems can indeed be used to spatially control a bioink location within a 3D complex tissue. This precise spatial control could be paired with other technologies, such as 3D mapping of native tissues or embryonic development [133], to print patient-specific tissues containing identical cellular and ECM components in their respective locations and densities. The combination of these multiple technologies makes it possible for the planned-printing of tissues to optimize fusion and self-assembly, based off results gathered from studying embryonic and organ development. Overall, results suggest that a comprehensive understanding of printing parameters, bioink deposition and fusion properties, and tissue mechanical properties is necessary before a procedure for printing both organs and tissues becomes possible.
Figure 1.3. Scaffold-Free Vascular Grafts. Aside from permanent or degradable polymeric scaffolds, vascular grafts are also fabricated using biological sources. These techniques include cell sheets, cell aggregates, and tissue printing strategies. Methods have been developed in which a sheet of cells is physically wrapped around a mandrel,
followed by culture and eventual decellularization of the cell tube to leave behind ECM [95]. This ECM can then be seeded with autologous cells prior to implantation. Although successful in early clinical trials, this technique is both costly and time consuming. Additionally, cells may be seeded into a custom mold to promote cell fusion and assembly of cellular aggregates. While these tissues can be assembled rapidly and with controlled geometry and composition, difficulties often arise with low mechanical properties and tissue necrosis [103]. Finally, tissues can be printed using custom inkjet printers or bioink & biopaper combinations [132, 134]. While such strategies offer rapid fabrication times and precise control of vessel geometry and composition, they are in the developmental stage, often requiring maturation prior to use and encountering difficulty matching required mechanical properties.

1.4. Nanotechnology in Tissue Engineering

The use of nanotechnology for applications in tissue engineering has been utilized due to the integrative methods with which nanomaterials can be applied to tissue engineering [135-139]. The main goals of incorporating nanotechnology with tissue engineering entail mimicking nanostructures of the native tissue and to integrate functionalities that conventional tissue engineering cannot address [110]. Typical methods to integrate nanotechnology into tissue engineering involve nanolithography, nanowires, nanofibers, nanoparticles, surface coating, and drug delivery (Figure 1.4) [140]. Nanolithography allows for the control of physical surface properties at the nanometer level. This is useful in vascular tissue engineering as it can modify the surface of a graft to closely mimic the native extracellular matrix, as the ECM has been shown to regulate cellular functions such as morphogenesis, differentiation, proliferation, adhesion, and migration [141]. Studies have shown that surface patterning and topography are important in cell adhesion and differentiation, with materials possessing nanopatterned surfaces demonstrating improved cell adhesion and function (endothelial, smooth muscle) compared to unpatterned materials [142-144]. Additionally,
investigations into a variety of materials (collagen, elastin and polymers such as PLLA, PLGA, and PCL) have been undertaken to determine their suitability as nanofibers for scaffold materials in vascular tissue engineering. Indeed, electrospun nanofiber scaffolds composed of 45% (by weight) collagen, 15% elastin, and 40% biodegradable polymer have already been fabricated that mimic the native ratio of collagen and elastin in blood vessels [92]. Nontoxic scaffolds, with fibers 477-765 nm in diameter, exhibited significant cell infiltration throughout the scaffold after 21 days in culture. Electorspun small diameter vascular grafts have demonstrated high long-term patency rates in vivo with noticeable tissue integration and scaffold degradation over time [145, 146]. Nanotechnology has also been applied to vascular grafts to locally deliver bioactive factors including growth factors, drugs, antibodies, and genes [147-149]. Overall, nanotechnology is applicable to vascular tissue engineering in a variety of methods to improve tissue integration and functionality. Though in vivo results have been promising, further investigation is necessary to fully elucidate these nano-scale interactions [94, 110, 150, 151].
Figure 1.4. Applications for Nanotechnology in Tissue Engineering. Nanotechnology has been the subject of recent research for integration into tissue engineering constructs due to its ability to mimic the native ECM and cellular environment. Strategies employed include nanolithography, nanowires, nanofibers, nanoparticles, surface coating, and drug delivery across a variety of tissue engineering fields [140]. Nanolithography has been applied to modify the surface geometry of tissue scaffolds to promote, deter, or control cell and protein interactions [142, 144]. Nanofibers can be used to closely mimic the small scale of ECM fibers throughout the cell and its native environment, thereby closely mimicking the surrounding environment and promoting cell integration [145,
Additionally, chemical modifications can be made to the scaffold to either coat the surface to control biological interactions, or to load scaffolds with drugs or growth factors to promote integration [148, 149].

1.5. Magnetic Nanoparticles in Tissue Engineering

Magnetic nanoparticles (MNPs) have gained interest in biomedical applications due to their complementary and multifunctional capabilities in imaging, drug delivery, and magnetic targeting, with some iron oxide MNP formulations currently FDA approved and serving as treatments for iron deficiency (Feraheme) or MRI contrast agents (Feridex) [152-154]. MNPs can be modified to allow researchers to control MNP interaction within the body at sub-cellular levels. Furthermore, MNPs can serve multiple simultaneous functions (i.e. imaging agent and targeted drug delivery), allowing for more effective treatment modalities. Currently, MNPs have been investigated for applications in magnetic hyperthermia for cancer therapy [155, 156], magnetic cell separation for diagnostics [157, 158], in vivo cell tracking for imaging [159, 160], and even in vivo monitoring of transplanted tissues [161, 162]. However, the prolonged presence of MNPs can have adverse effects on cells, such as cell toxicity, changes in cell phenotype, or changes in cell mobility [163-165]. Surface functionalization or coating of MNPs in materials such as oleates [166, 167], dextran [154, 168], or polymers [162, 169] can enhance MNP biocompatibility and functionality by improving cell viability, uptake, or biodistribution. These techniques often involve complex chemistry and do not necessarily accelerate the removal of MNPs from the body. Therefore, methods to alter MNPs so their presence in the body is limited will benefit biomedical MNPs. Ideal
biomedical MNPs would remain stable for sufficient time to accomplish their desired role and rapidly degrade once their task is completed.

Iron oxide MNPs are ideal for tissue engineering applications due to their superparamagnetic properties and ability to fabricate uniform particle sizes that can be functionalized to suit desired requirements. However, a primary concern with biomedical MNPs is their fate within the body. Following systemic administration, larger iron oxide MNPs (>200 nm diameter) are sequestered by the spleen as a result of mechanical filtration [170]. These MNPs are ultimately removed from the cells through phagocytosis, while MNPs with small diameters (<10 nm) are rapidly cleared through extravasations and renal clearance. Iron oxide MNPs, once internalized into cells, are directed to lysosomes where the low pH and enzymatic activity metabolize the nanoparticle into iron and oxygen [171, 172]. Intercellular enzymes work to catalyze the oxidation of Fe(II) to Fe(III), which can be bound to transferrin, an iron binding glycoprotein that regulates the level of free iron in biological fluids and tissues [173]. The biodistribution and pharmacokinetics of MNPs are also a concern in biomedical applications. Following intravenous injection, Jain et al. showed that MNPs primarily localized in the liver and spleen, but MNPs were also found in the brain, heart, kidneys, and lungs [174]. Roughly 55% of the injected iron was localized in the liver 6 hours after injection, with 50% and 25% of the injected iron still present in the liver and spleen after 3 weeks, respectively. Furthermore, their results also confirm that the injected iron oxide does degrade over time, with magnetization measurements of the liver and spleen showing a steady decrease over 3 weeks. Studies have confirmed that portions of
injected MNPs are excreted through urine and feces, with some formulations showing as much as 35% after 28 days [175] while others demonstrate 18-22% excretion after 7 weeks [176]. However, methods that can accelerate this MNP degradation will limit the interaction of MNPs with the biological environment, which is expected to be critical to reduce cytotoxicity.

A variety of different chemicals have been investigated to accelerate the degradation of iron oxide MNPs. The ability to increase the degradation rate of MNPs within the body will allow for decreased exposure of cells and the body to these MNPs, and thereby limit their interaction. Multiple acids have been confirmed to dissociate iron oxide, including oxalic, citric, lactic, and acetic acids [177]. Miller et al. confirmed that of thirteen acids tested, oxalic and citric acid (0.1 M) lead to complete dissociation of iron oxide over 100 hours at pH 3.5 at room temperature, with less complete dissociation occurring at pH 5.5 compared to pH 3.5. Lactic acid also led to dissociation of iron oxide, demonstrating roughly 80% degradation at pH 3.5, but only 10% degradation at pH 5.5. However, other studies have shown that exposure of cells to pH 5.7 or below using acetic and lactic acid (0.12-0.16 M) induced toxicity on cells [178]. These results highlight that while acids can be used to induce degradation of iron oxides, the acidic environment required for iron oxide degradation can be detrimental to cells.

While it is clear that incubation in an acidic environment leads to accelerated degradation of iron oxide, a method to control this acidic environment such that its effects on cells are limited is needed for biomedical applications. Polymeric microparticles and nanoparticles can be utilized to create localized acidic environments
due to their degradation, which creates a local acidic microenvironment generated from
degradation byproducts including lactic and glycolic acid. Studies have confirmed that
degradation of PLGA(50/50) microparticles creates a spatial acidic environment within
degradation particles over the course of 15 days [179]. Microspheres formed using a
double emulsion technique revealed a pH of 1.5 within the core of microparticles, but
maintained a pH greater than 3.5 on the edge of particles. Utilization of low
concentrations of polymeric particles will not effect the overall pH of the
macroenvironment due to a localized decrease in pH in the particle microenvironment.

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CHAPTER 2

JANUS MAGNETIC CELLULAR SPHEROIDS

2.1. Introduction

Three dimensional cell cultures, or spheroids, have been investigated as tissue engineered building blocks due to their ability to mimic the native cellular and extracellular matrix (ECM) environment. Methods to pattern cellular spheroids include direct seeding into a mold [1, 2], printing into hydrogel or biopaper [3-5], or surface patterning to promote adhesion [6-9]. Nanotechnology has emerged as pivotal part of biomedical engineering practices, with successful applications in imaging [10, 11], drug delivery [12, 13], biosensors [14, 15], and tissue engineering [8, 16-19]. By tailoring and controlling interaction at a sub-cellular level, nanotechnology can be used to improve biocompatibility, tissue integration, and assembly of complex tissues or scaffolds [9, 20, 21]. The use of magnetic forces to manipulate and align cells or cellular spheroids can be utilized for this patterning and expanded into tissue engineering [22-28]. A variety of methods that utilize internalization of MNPs into cells have been investigated to integrate MNPs with tissue engineering, including MNP conjugation/binding to the cell [24, 28, 29], cellular internalization [30-33], and mixing MNPs within the ECM [22, 30, 34]. These methods typically produce adverse effects related to MNP uptake into cells, however, highlighting the need to limit MNP and cell interaction. In the presented approach, we go beyond current MNP approaches by reducing MNP interactions with cells, thereby avoiding adverse effects [22, 23, 29, 33, 35], while still allowing for magnetic manipulation. These novel structures possess two segregated domains, one
composed of cells and the other of extracellular MNPs to form a Janus magnetic cellular spheroid (JMCS, Figure 2.1). The ability to magnetically assemble engineered building blocks that maintain long-term cell viability and stable phenotype is critical for incorporating MNPs into tissue structures. To our knowledge, this is the first report of JMCS structure as building blocks for tissue engineering.

![Figure 2.1. Methods to Incorporate MNPs into Cellular Spheroids](image)

**Figure 2.1. Methods to Incorporate MNPs into Cellular Spheroids.** Janus magnetic spheroid structures do not exhibit the cytotoxic affects associated with MNP internalization common to dispersed and uptake methods by separating MNPs and cells into segregated and distinct regions. The combination of MNPs (black), cells (pink), and ECM (collagen, blue) into segregated regions decreases cellular toxicity related to MNPs by decreasing interactions with cells.

**2.2 Materials & Methods**

An overview of experimental procedures, parameters, and objectives can be found in Table 2.1.
Table 2.1. Experimental Plan for Janus Magnetic Cellular Spheroid Analysis.
Experiments were performed to compare JMCSs to common Uptake magnetic cellular spheroids, histologically analyze the segregation of MNPs and cells within JMCSs, determine factors affecting JMCS viability, determine the effect of MNPs on SMC phenotype expression within JMCSs, and determine the effect of MNPs on collagen synthesis within JMCSs. Multiple parameters were varied within each experiment as noted.
2.2.1. Statistical Analysis

All statistical analysis was performed using a two-tailed t-test with at least three repeats each. Statistical significance was set at $p < 0.05$. Error bars on graphs represent the standard deviation from the mean.

2.2.2. Cell Culture

Primary rat aortic smooth muscle cells (SMCs), primary rat aortic fibroblasts (FBs), and human adipose-derived stem cells (ADCSs, Lonza) were used for all studies. All cells were cultured in monolayer cultures at 37 °C and 5% of CO$_2$ until spheroid assembly. SMCs were cultured using Dulbeco’s Modified Eagle Medium:F-12 (ATCC, 1:1, DMEM:F-12) supplemented with 10% fetal bovine serum (Atlanta Biologics) and 1% penicillin-streptomycin-amphotericin (MediaTech, Inc.). FBs were cultured using Dulbeco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin. ADSCs were cultured using adipose-derived stem cell basal medium (Lonza) supplemented with 10% fetal bovine serum, 1% L-glutamine (Lonza), and 0.1% gentamicin-amphotericin B (GA-1000, Lonza).

2.2.3. Spheroid Assembly

To assemble JMCSs, iron oxide MNPs (Fe$_3$O$_4$, 20 – 30 nm, SkySpring Nanomaterials, Inc.), collagen (Bovine, Type I, Life Technologies), and cells in cell culture media were dispensed using a modified hanging drop method into 15 µL droplets [24]. Samples were inverted and incubated at 37 °C with 5% CO$_2$ for three days prior to use to allow for spheroid assembly. Specific details on the modified method cannot be disclosed at this time. Unless otherwise noted, all spheroids were assembled using SMCs.
with MNPs, 20,000 cells per spheroid, and collagen. To assemble uptake MNP spheroids, a monolayer cell culture flask (~90% confluence) was incubated with MNP-containing cell culture media (300 µg/mL) for 24 hours. The bottom of culture flasks was covered with square magnets (K&J Magnetics, Inc., 12.7 x 12.7 mm, 1.6 mm thick, vendor calculated pull force = 3.59 lbs) to promote MNP internalization into cells. Media solutions containing MNPs were sonicated prior to addition to cells. After incubation, cells were washed 5x to remove free MNPs, trypsinized (0.25%, Thermo Scientific), collected, and placed on a magnetic wash tool and allowed to sit for 5 minutes. The supernatant was discarded and remaining magnetically attracted cells suspended in fresh media. Solutions of magnetically attracted cells and collagen were combined and dispensed using a hanging drop method as mentioned previously. Collagen was prepared according to manufacturer recommendations and kept on ice prior to use for all samples. MNPs were washed three times with a magnetic wash tool prior to use to remove byproducts.

2.2.4. Histology

JMCSs were processed and sectioned via standard paraffin sectioning techniques. Briefly, spheroids were fixed with Z-Fix (buffered zinc formalin, Anatech Ltd.) and dehydrated using ethanol and xylene prior to paraffin embedding. 5 µm thick sections were stained using hematoxylin and eosin (H&E) and Masson’s Trichrome (Poly Scientific). JMCSs were collected and analyzed after three days of assembly in hanging drop.
2.2.5. Viability

PrestoBlue (Life Technologies) and MTT (Sigma) cell viability assays were performed to quantify cell viability (at least 3 repeats per sample). Spheroids were dissociated via incubation with collagenase (Collagenase Type IV, Life Technologies). Spheroids were centrifuged and physically dissociated, then allowed to adhere overnight on a well plate. Viability was also qualitatively analyzed following manufacturer’s specifications (Life Technologies) using simultaneous live / dead fluorescent staining and imaging using Calcein, AM (live) and ethidium homodimer-1 (EthD-1, dead). Spheroids were stained, fixed using Z-fix, and imaged using a Nikon Eclipse Ti confocal microscope. All JMCSs were collected and analyzed after three days of assembly in hanging drop except those used for long-term viability studies. Spheroids used for long term viability studies were cultured in non-treated well plates with media changes every other day.

2.2.6. Quantification of MNP Internalization

First, cellular spheroids (25, Janus and Uptake) were dissociated. Dissociated samples were suspended in 1 mL fresh media and placed on a magnetic wash tool for five minutes. The supernatant was collected, and the remaining cells (magnetically attracted) suspended in 1 mL fresh media. The amounts of cells in the supernatant and magnet solution were quantified using a hemocytometer. Spheroids were collected and analyzed after three days of assembly in hanging drop.
2.2.7. Phenotype Analysis

Flow cytometric analyses were conducted using a Beckman Coulter Astrios cell sorter. Spheroids were dissociated, filtered, suspended in CytoFix/Cytoperm buffer, and incubated with primary antibodies for 30 minutes at 4 °C. Three SMC markers were analyzed: smooth muscle α-actin (SMAA) conjugated with Cy3 (Sigma-Aldrich), smooth muscle 22 (SM22, Abcam), and smooth muscle myosin heavy chain (SMHC, Biomedical Technologies, Inc.). Antibodies used include: SMAA – mouse monoclonal anti-actin, α-smooth muscle – Cy3 antibody, SM22 – rabbit polyclonal anti-SM22 alpha antibody, SMHC – rabbit anti-smooth muscle myosin IgG antibody. Samples were washed 3x with Cytowash buffer and incubated with secondary antibodies for 30 minutes at 4 °C. Next, samples were washed once with Cytowash buffer, followed by one wash in FACs buffer (1% bovine serum albumin in PBS). Samples were resuspended in FACs buffer and analyzed via flow cytometry. Rabbit IgG-Cy5 (Jackson ImmunoResearch) was used as a secondary antibody.

Immunofluorescence microscopy was used to qualitatively confirm SMC phenotype expression within samples. First, OCT embedded histology sections (5 µm thick) were collected and cleared via two washes (5 minutes) in PBS and 1 wash in water. Samples were circled with a diamond tip pen. Samples were treated with a 0.1% Triton X-100 to permeabilize samples (10 minutes). Slides were washed 3x with water (5 minutes), followed by a 30 minute incubation with Background Buster (Innovex Biosciences). Next, samples were washed 2x water and 1x PBS. Once samples were prepped, they were incubated with primary antibodies overnight at 4 °C in a humid
chamber. Samples were washed 3x PBS, followed by incubation with secondary antibodies for one hour at room temperature. As a control, samples were incubated with just the secondary antibody to ensure controlled binding was occurring. Finally, samples were washed 3x PBS followed by incubation with Hoechst 33342 to stain for nuclei (10 minutes, room temperature). Samples were washed 3x PBS and cover slipped, and fluorescently imaged for SMHC and SM22 (FITC, Ex/Em 492/520), SMAA (Cy3, Ex/Em 552/570), and nuclei (Hoescht 33342, Ex/Em 343/483). Results were analyzed and compared to samples containing no MNPs to determine the effects of MNP integration on SMC phenotype marker expression. Three samples of each formulation were analyzed to confirm that results were consistent. Spheroids were collected and analyzed after three days of assembly in hanging drop.

2.3. Results & Discussion

2.3.1. Janus vs. Uptake Magnetic Cellular Spheroids

To analyze the spatial localization of MNPs and cells within JMCSs, samples with Janus structures were histologically sectioned and stained using Masson’s Trichrome (Figure 2.2A) and hemotoxylin and eosin (H&E, Figure 2.2 B-E). Samples containing MNPs showed localization of MNPs and cells into distinct regions. Overall, the concept of using magnetic forces to manipulate cellular spheroids has been shown to be promising, but the advances to integrate MNPs with tissue fabrication have failed due to toxicity related to MNP uptake into cells or adverse affects on cell function and morphology [33, 36, 37]. Ultimately, a magnetic cellular spheroid structure possessing
two segregated domains, one composed of cells and one composed of MNPs, will fully incorporate the positive benefits of using magnetic cellular spheroids to go beyond current approaches. The formation of separate domains reduces the interaction and uptake of MNPs into cells, thereby avoiding any adverse effects while still allowing for magnetic force manipulation.

![Figure 2.2. Structure of Janus Magnetic Cellular Spheroids.](image)

(A) The separation of MNPs and cells is present within JMCSs containing dispersed collagen and cells with a segregated MNP domain (Masson’s Trichrome) (B-E) Samples were stained using H&E to confirm the presence and segregation of MNPs within cellular spheroids, (No MNPs = B, increasing MNP concentrations C-E, scale bars = 100 µm). Results confirm the segregation of MNPs (black) and cells (purple) into distinct domains using multiple MNP concentrations.

Next, JMCSs were compared to Uptake MNP spheroids to determine if the Janus structure improved upon MNP interaction with cells within cellular spheroids. The amount of cells that internalized MNPs were quantified and showed that JMCSs resulted in significantly lower internalization (35%) compared to uptake spheroids (83%, p < 0.05, **Figure 2.3A**). Next, cellular viability using both Janus and Uptake cellular spheroids was analyzed up to one week to determine their capacity for tissue engineering.
applications which involve prolonged interaction between MNPs and cells. A viability analysis (300 µg/mL MNP) also showed that JMCSs maintained high viability comparable to control spheroids without MNPs, while uptake spheroids resulted in lower viability at Days 3 and 7 (p < 0.05, Figure 2.3B). Finally, the use of JMCSs was compared to conventional uptake magnetic cellular spheroids for tissue assembly (Figure 2.3C). Spheroids were assembled onto a magnetic pattern and allowed to fuse for 48 hours, at which point the magnet was removed (Figure 2.3C, before), and the rings were transferred to new a chamber. Fused rings composed of Janus spheroids maintained tissue structure in contrast to those composed of uptake spheroids, which dissociated upon handling (Figure 2.3C, after). Based off viability results, this dissociation is due to decreased cell viability, and therefore tissue integrity, of Uptake magnetic cellular spheroids. Results confirm that the method used to incorporate MNPs into cellular spheroids is critical, as internalization of MNPs clearly has an effect on cell viability.
Figure 2.3. Janus vs. Uptake Magnetic Cellular Spheroids. (A) The amount of cells per spheroid that internalized MNPs was quantified and revealed that JMCSs (35%) resulted in significantly less MNP internalization than uptake cellular spheroids (83%, * p < 0.05). (B) Additionally, cell viability analysis of cellular spheroids fabricated using Janus and uptake methods revealed that JMCSs maintained high viability compared to control spheroids without MNPs, while uptake spheroids exhibit low viability (* p < 0.05) at Days 3 and 7. (C) The use of both spheroid types for tissue engineering applications was analyzed by assembling fused rings via magnetic patterning. After 48 hrs of fusion, the magnetic patterns were removed and the rings transferred to another chamber. Results revealed that rings assembled using uptake spheroids unable to be physically manipulated and broke apart upon handling. Rings assembled using Janus spheroids, however, were capable of handling, and therefore applicable to tissue engineering applications (scale bars = 1000 µm).
2.3.2. Factors Affecting Cell Viability

To determine the capacity for JMCSs to be utilized across a variety of tissue engineering applications, quantitative and qualitative methods were used to analyze viability using various JMCS formulations. Cell density, ECM content and ECM composition have been demonstrated to be critical to tissue formation and fabrication [4, 38, 39]. First, the effect of JMCS composition on cell viability was analyzed by varying the length of culture (Figure 2.4A,B) and on different cell types (Figure 2.5A). Next, using rat aortic smooth muscle cells (SMCs) and rat aortic fibroblasts (FBs), the effect of JMCS composition on spheroid viability was analyzed. JMCSs were fabricated to determine the effects of various collagen I and elastin contents (Figure 2.5B,C), collagen I and elastin ratios (Figure 2.5D), and cell densities (Figure 2.5E) on spheroid cellular viability. For all formulations, the MNP concentration was held constant at 0.3 mg/mL. Cell viability was quantified up to seven weeks, with results demonstrating sustained high viability over the seven weeks compared to cellular spheroids without MNPs (Figure 2.4A,B). Statistical analysis on long-term viability samples showed significant differences between samples at Day 3 and 1 Week, but in favor of JMCSs compared to controls (normalized viability greater than 100%). Though a statistically significant difference was noted at 5 Week (p < 0.05), this difference disappeared at Week 7. Spheroid viability was also qualitatively assessed at 7 weeks using simultaneous live/dead staining, confirming equivalent expression in spheroids with and without MNPs (Figure 2.4B). These results confirm the long-term in vitro viability of JMCSs. Second, we prepared JMCSs using three different cell types to determine if cell viability was
dependent on cell type (Figure 2.5A), which could prevent further applications to fabricate multicellular tissues. Compared to samples without MNPs, JMCSs composed of rat aortic smooth muscle cells, human adipose derived stem cells (ADSCs), and rat aortic fibroblasts maintained high cell viability. Next, the effect of JMCS composition on viability was analyzed up to one week using SMCs and FBs. Results showed that collagen I concentrations ranging from 0.01 mg/mL to 1.3 mg/mL had no adverse effects on cell viability when compared to spheroids without collagen I (Figure 2.5B). Additionally, elastin concentrations ranging from 0.005 mg/mL up to 0.65 mg/mL maintained high viability when compared to control spheroids without elastin (Figure 2.5C). Next, holding the total ECM content constant (0.24 mg/mL), elastin to collagen ratios were varied to mimic variations in ECM between different tissue types. Results showed that various elastin to collagen ratios from 0.3 to 1.5 had no adverse effects on spheroid viability when compared to spheroids without ECM (Figure 2.5D). Finally, the density of cells within JMCSs was varied, ranging from 20,000 to 100,000 cells per spheroid. High viability was maintained at all cell densities when compared to spheroids without iron oxide MNPs (Figure 2.5E). High viability was maintained for both cell types tested, SMCs and FBs, for all JMCS formulations. Viability was also maintained in short term studies with MNP concentrations up to 5 mg/mL (Figure 2.6), demonstrating a decreased cytotoxicity compared to previous studies [23, 32, 33, 36, 37] primarily due to the nature of the JMCS’s segregated domains. Additionally, the ability of JMCSs to maintain high viability across a variety of ECM and cellular compositions makes them appealing for tissue engineering applications due to their capacity to be used for the
assembly of various tissue types. Additionally, groups have shown that cell density is critical to fabricating tissue constructs, as higher cell density spheroids have lead to increased viability and metabolic output, compared to lower cell densities [40]. This novel method for MNP-incorporation is expected to overcome the problems associated with MNP internalization, thereby allowing for more complex and long-term applications using MNPs in tissue engineered constructs. The ability to magnetically assemble tailored engineered building blocks with controlled cell and ECM compositions provides a capability to assemble tissue structures with tailored compositions.

**Figure 2.4. JMCS Viability – Long Term.** (A,B) Results demonstrated that using Janus magnetic cellular spheroids structures, long term viability was maintained up to 7 weeks, confirmed both qualitatively (confocal microscopy, live/dead stain) and quantitatively compared to spheroids without MNPs (scale bars = 500 µm). Statistical analysis showed significant differences between Janus MNP and control cellular spheroids without MNPs at day 3, 1 week, and 5 week (* p < 0.05).
Figure 2.5. JMCS Viability – Varying Cellular and ECM Compositions. (A) Viability of JMCSs was not affected by cell type used, with high viability maintained using human adipose-derived stem cells (ADSCs), rat aortic fibroblasts (FBs), and rat aortic smooth muscle cells (SMCs). Furthermore, results demonstrate high viability of
(B) varying collagen, (C) varying elastin, (D) varying elastin:collagen ratios, and (E) varying cell number in JMSCs up to one week. High viability was maintained using both smooth muscle cells (SMCs) and fibroblasts (FBs) for collagen concentrations up to 1.3 mg/mL, elastin concentrations up to 0.65 mg/mL, varying elastin/collagen ratios, and at various cell densities. Spheroids were compared to control spheroids without the respective ECM component (collagen, elastin) or iron oxide MNPs (varying cell number). When varying collagen and elastin content, some samples from both cell lines demonstrated statistically significant differences compared to controls with no ECM (* p < 0.05), but all samples maintained greater than 90% viability and were therefore considered to maintain high viability. Additionally, when varying cell number, some samples demonstrated significant differences compared to control samples without MNPs. However, all samples maintained greater than 90% viability, and were therefore considered to maintain high viability.

Figure 2.6. Effect of Varying MNP Concentrations on JMCS Viability. Viability analysis confirmed no statistically significant differences in viability at Day 3 when comparing spheroids with varying MNP concentrations to those without MNPs (t-test, p > 0.05, three repeats each).

2.3.3. Factors Affecting Cell Phenotype

Once viability was confirmed, we analyzed samples to determine any adverse effects of MNPs on cell phenotype expression. Flow cytometric analysis was performed on cells from spheroids (control with no MNPs, Janus MNPs, and Uptake MNPs) to
determine the percentage expression of three SMC markers: smooth muscle α-actin (SMAA), smooth muscle 22 (SM22), and smooth muscle myosin heavy chain (SMHC, Figure 2.7A). Compared to no MNP control spheroids, JMCSs, and Uptake MNP spheroids maintained equivalent expression of SMAA (99% and 93% respectively, normalized to control). Additionally, high SM22 expression was maintained for Janus spheroids (95%), while Uptake spheroids resulted in decreased expression (74%). However, the addition of MNPs using the Janus method did result in a slight decrease in SMHC expression compared to no MNP controls (75%), while Uptake MNP spheroids exhibited a significant decrease in expression (16%). These results were also qualitatively analyzed using immunofluorescence microscopy (Figure 2.7B), with a uniform dispersion of markers throughout JMCSs compared to controls without MNPs. These results indicate that the presence of MNPs within Janus spheroids did not affect cell phenotype, compared to spheroids without MNPs as both populations expressed SMC-specific markers at similar levels. Although some studies have shown that MNPs have no adverse effects on cell phenotype [34, 41], others have demonstrated adverse impact on cell motility, length, and other morphological abnormalities, suggesting that use of MNPs results in altered cell phenotype [36, 37]. Consequently, it is critical to prevent MNP interactions with cells for tissue engineering applications.
Figure 2.7. Effect of MNPs on Cellular Phenotype. (A) SMC phenotype expression of three known SMC markers was quantified using flow cytometry (at least 1 million cells analyzed per sample). Results indicate that JMCSs exhibited similar expression of all three markers compared to controls, while uptake MNP spheroids results in decreased expression of SM22 and SMHC. (B) Phenotype expression was qualitatively confirmed using IHC microscopy (red and green = marker, blue = nucleus), confirming uniform dispersion and equivalent expression levels of SMC phenotype markers in JMCSs compared to control spheroids without MNPs.

2.3.4. JMCSs for Tissue Engineering – Collagen Synthesis

Collagen occupies the extracellular space and regulates cellular activity and tissue function by providing a natural scaffold for controlling the spatial arrangement of cells within tissues [42, 43]. An appealing aspect of cellular spheroids for tissue engineering
applications is their ability to dynamically produce their own ECM to meet required mechanical or functional needs of the local environment [2]. Therefore, experiments were performed to provide evidence of this phenomenon and to confirm that using JMCSs, the addition of iron oxide MNPs has no adverse effects on collagen synthesis. First, we demonstrated that JMCSs could be tailored to contain controlled ECM contents, as confirmed using collagen for a proof of concept (Figure 2.8A). Using Masson’s Trichrome stain to visualize collagen, these results demonstrated that collagen content can be tailored in the fabrication process. Next, spheroids were histologically sectioned and stained to visualize collagen production up to Day 40 (Figure 2.8B). Initial collagen concentrations were held constant at 0.017 mg/mL for spheroids with and without MNPs. Results demonstrated that JMSCs and MNP-free spheroids secrete their own collagen, as confirmed by an increase in collagen within cellular spheroids over time. Furthermore, visual analysis suggested that the addition of MNPs to cellular spheroids lead to increased collagen production compared to MNP-free spheroids. These results suggest that the presence of MNPs within cellular spheroids has no adverse effects on collagen synthesis, and that MNPs may stimulate accelerated collagen synthesis compared to MNP-free controls. Therefore, JMCSs are a viable candidate for tissue engineering applications due to their ability to secrete their own ECM as the engineered tissue develops. Collagen and elastin are the two most important matrix proteins that mediate the mechanical properties of tissues [38, 43]. Thus, the development of a viable tissue engineered construct with mechanical properties similar to native tissues heavily relies on enhancing collagen and elastin production.
Figure 2.8. Effect of MNPs on Collagen Synthesis within JMCSs. (A) Collagen content can be controlled in the fabrication process, confirmed using Masson’s Trichrome stain. Visual analysis confirms an increase in collagen (blue) content as the initial collagen content is increased during the JMCS fabrication process. (B) JMCS and MNP-free spheroids both secrete their own collagen over time, confirming that MNPs have no adverse effects on collagen synthesis within cellular spheroids. Using Masson’s Trichrome, this was visually confirmed by the increase in collagen (blue) over time up to Day 40. Scale bar = 500 µm.
2.4. Conclusions

Results indicate that while MNPs can be incorporated into cellular spheroids using a variety of different techniques, they can induce adverse effects on cells. However, the method in which MNPs are incorporated into cellular spheroids is critical, as Janus structures reduce adverse effects seen in Uptake MNP cellular spheroids. Cytotoxicity and phenotypic analyses showed that the JMCS structure can integrate MNPs with biological structures without compromise to cells, thus extending beyond current approaches using MNPs to engineer heterogeneous constructs composed of multiple cell lines. Due to the requirement of long-term cell and MNP interactions to prepare fully functional complex tissues, it is critical to maintain long-term cell viability and phenotype. Furthermore, JMCSs have no adverse effects on collagen synthesis within cellular spheroids, making them ideal candidates for tissue engineering applications due to the mechanical support provided by collagen.

2.5. Works Cited


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CHAPTER 3

SPHEROID FUSION USING MAGNETIC FORCE ASSEMBLY

3.1. Introduction

Scaffold-free cellular aggregates produce and secrete their own extracellular matrix (ECM) and can fuse into complex tissue structures [1-4]. Fabrication of functional tissue engineered constructs with appropriate cell densities, ECM contents, and mechanical properties that mimic native tissues still remain a challenge due to the lack of understanding associated with tissue fusion and maturation mechanisms [3, 5]. A critical process in the fabrication of complex tissue structures with cellular spheroids is related to their fusion [4]. Tissue fusion is a self-assembly process in which two or more distinct cell populations, or tissues, make contact and coalesce to form a single cohesive structure [2-4]. Though the process is not clearly understood, research has shown that factors mediating tissue fusion include cell migration, cell-cell interactions, and cell-matrix interactions [2, 4, 6]. Tissue fusion is driven by minimizing the overall system configurational energy, which results in smaller tissue aggregates [2, 7].

By incorporating magnetic nanoparticles (MNPs) into cellular spheroids, tissues can be aligned and patterned using magnetic force assembly [8-14]. Furthermore, magnetic forces can mediate and enhance tissue fusion by promoting cell-to-cell contacts and interactions. These increased interactions arise from the adhesive and cohesive interactions between cells under the influence of magnetic attraction. Conventional non-magnetic tissue assembly and fabrication methods include cell printing, cell sheet techniques, and patterned molds [2, 3, 15, 16]. These non-magnetic methods spatially
orient the cells into a desired position through passive contact, but do not address active contact mediated by forces. Therefore, Janus magnetic cellular spheroids (JMCSs) were used as a model for spheroid fusion mediated by magnetic forces. Results demonstrated that magnetic forces play a large role in mediating spheroid fusion when using magnetic force assembly. An understanding of the role of MNPs and magnetic force in mediating spheroid fusion can lead to optimization of tissue patterning and printing to promote fusion in a desired direction.

3.2. Materials & Methods

An overview of experimental procedures, parameters, and objectives can be found in Table 3.1.
### Table 3.1. Experimental Plan for Analysis of JMCS Fusion into Complex Tissues

Experiments were performed to analyze the ability to assemble fused tissues using JMCSs and magnetic force assembly. Furthermore, studies were performed to determine factors mediating JMCS fusion into complex tissues, and the capacity of JMCSs to be used for the assembly of a complex multicellular vascular tissue construct. Multiple parameters were varied within each experiment as noted.

#### 3.2.1. Statistical Analysis

All statistical analysis was performed using a two-tailed t-test with at least three repeats each. Statistical significance was set at $p < 0.05$. Error bars on graphs represent the standard deviation from the mean.
3.2.2. Cell Culture

Primary rat aortic smooth muscle cells (SMCs), primary rat aortic fibroblasts (FBs), and rat aortic endothelial cells (ECs, Cell Applications, Inc.) were used for all studies. All cells were cultured in monolayer cultures at 37 °C and 5% of CO₂ until spheroid assembly. SMCs were cultured using Dulbecco’s Modified Eagle Medium:F-12 (ATCC, 1:1, DMEM:F-12) supplemented with 10% fetal bovine serum (Atlanta Biologics) and 1% penicillin-streptomycin-amphotericin (MediaTech, Inc.). FBs were cultured using Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin. ECs were cultured using Rat Endothelial Cell Growth Medium (Cell Applications, Inc.).

3.2.3. Spheroid Assembly

To assemble JMCSs, iron oxide MNPs (Fe₃O₄, 20 – 30 nm, SkySpring Nanomaterials, Inc.), collagen (Bovine, Type I, Life Technologies), and cells in cell culture media were dispensed using a modified hanging drop method. Unless otherwise noted, all spheroids were assembled using SMCs with MNPs, 20,000 cells per spheroid, and collagen.

3.2.4. Magnetic Patterning

To assemble various shapes using magnetic force assembly, commercial magnets (SuperMagnetMan, Birmingham, AL) of various shapes and strengths were applied to the bottom of glass chamber slides and maintained for four days. All shapes were allowed to fuse for four days with the magnet in place, with changes in cell culture media every other day. Magnets were removed on the fourth day, and fused tissues were immediately
imaged using a Nikon AZ100 multizoom microscope. Small rings were assembled using ring magnets (2 mm OD, 1 mm ID, 1 mm thick, vendor calculated pull force = 0.16 lbs). Medium and large rings were assembled using aligned cylinder magnets in a circular pattern (2.5 mm diameter, 5 mm length, pull force = 1.8 lbs). Square shapes were patterned using aligned cylinder magnets in a square pattern (2.5 mm diameter, 2.5 mm length, pull force = 1.57 lbs). The Clemson University Tiger Paw shape was assembled using custom cut magnetic strips. All fusion studies were performed with SMC JMCSs composed of 0.017 mg/mL collagen, 0.3 mg/mL iron oxide MNPs, and 20,000 cells.

3.2.5. Tracking Ring Fusion

Spheroid fusion was analyzed by tracking the fusion of spheroids into a ring (Figure 3.1). Ring magnets were commercially purchased (2 mm OD, 1 mm ID, 1 mm thick, vendor calculated pull force = 0.16 lbs). 25 individual SMC JMCSs (0.017 mg/mL collagen, 0.3 mg/mL MNPs, 20,000 cells) were carefully patterned around the ring pattern in a monolayer formation. Magnets were kept in place for 48 hours and then removed, followed by imaging with a Nikon AZ100 multizoom microscope at respective time points. Magnet strength was varied by increasing the distance of the ring magnet away from JMCSs (coverglass spacers placed on the bottom of the chamber slides). Four measurements for each diameter (inner and outer) were recorded and averaged at each time point. Samples were normalized with themselves (based on initial inner and outer diameter measurements at 48 hours), as each sample was analyzed as the percent-initial diameter. At least three repeats were performed for each sample.
Figure 3.1. Studying Spheroid Fusion via Magnetic Force Assembly. Twenty-five JMCSs were magnetically patterned and fused into a tissue ring. Ring magnets were secured to the bottom of a chamber slide. Spheroids were magnetically patterned for 48 hours, at which point the ring magnets were removed and images were taken daily to track the contraction and fusion of tissue rings. To vary the magnetic field force, additional glass coverslides were placed on the bottom of chambers to increase the distance between the magnet source and JMCSs.

3.2.6. Tissue Tube Assembly

Using a polystyrene petri dish, a long rod magnet (45 mm) composed of stacked cylinder magnets (2.5 mm diameter, 5 mm length, pull force = 1.8 lbs) was secured to the bottom side of the petri dish (Figure 3.2). A glass slide was placed on the top surface of the petri dish to promote spheroid fusion. Two thousand JMCSs were added to the media-filled petri dish and assembled on top of the magnetic template. Care was taken to seed JMCSs evenly along the pattern. Magnetic cellular strips were incubated and allowed to
fuse over 4 days, with media changes every other day. Next, magnetic cellular strips were wrapped around a silicone tube (5 mm OD, 2.5 mm ID, Cole-Parmer) containing a magnet (2.5 mm diameter, 5 mm length, pull force = 1.8 lbs) within its lumen to form a tube tissue. To avoid oxidation of the magnet by culture media, the ends of the silicone tube were plugged with silicone rubber. The silicone tube was rolled over the top of the cellular strip to effectively wrap the strip around the tube. Samples were incubated in culture media to allow more fusion for 6 additional days. To assemble bilayer tubes, two separate magnetic cellular strips were assembled and wrapped around the same silicone mandrel in sequential order based on the desired spatial orientation. This same technique was applied to assemble trilayer tissue tubes. Single layer tissue tubes were assembled using SMCs JMCSs (2,000). Bilayer tissue tubes were assembled using an internal strip of SMC JMCSs (2,000) and an external layer of FB JMCSs (2,000). Finally, trilayer tissue tubes were assembled using an internal strip of EC JMCSs (1,5000), medial strip of SMCs (1,5000), and external strip of FBs (1,500). All JMCSs were composed of 20,000 cells, with 0.24 mg/mL collagen, and 0.3 mg/mL iron oxide MNPs.
Figure 3.2. Assembly of Tissue Tube using Magnetic Force Assembly. Spheroids were patterned, fused, and wrapped into a tube using magnetic manipulation. (A) First, JMSCs were patterned and allowed to fuse into a homogenous strip over the course of 4 days. (B) Next, a cylinder magnet was placed inside the lumen of a silicone tube with an outer diameter of 5 mm. (C) Finally, the fused tissue strip was wrapped around the silicone tube and allowed to fuse for an additional 6 days.

3.2.7. Fluorescent Labeling of Trilayer Tube

Three separate trilayer tubes were assembled, each containing one fluorescently labeled spheroid type. Cells were fluorescently labeled in suspension prior to spheroid assembly using a Vybrant CFDA SE Cell Tracer Kit (Life Technologies). JMCSs were assembled for three days in a modified hanging drop as previously described, with all JMCSs composed of 20,000 cells, 0.24 mg/mL collagen, and 0.3 mg/mL iron oxide MNPs. Trilayer tissue tubes were assembled as previously described (1,500 spheroids per tissue strip). However, fusion times were decreased in order to maintain fluorescent
signal for imaging. Spheroids were seeded onto a tissue strip and allowed to fuse for 24 hours, then wrapped around a silicone tube and allowed to fuse for an additional 24 hours. Samples were fixed overnight, then histologically processed and sectioned the following day. Tissues were permeabilized with Triton-X and stained with DAPI to visualize nuclei. Samples were imaged using a Nikon Ti Eclipse microscope.

3.3. Results & Discussion

3.3.1. Factors Affecting Spheroid Fusion

We evaluated the ability of JMCSs to fabricate tailored 3D cell constructs and tissues through a series of experiments utilizing magnetic assembly into a variety of shapes (Figure 3.3 A-C, E,F). We used magnetic force manipulation to assemble shapes with varying sizes including rings, a square, and Clemson University Tiger Paw. Results showed that fused tissues, confirmed by a lack of voids or gaps within tissues, were successfully assembled using variety of shapes and sizes after four days of fusion using magnetic force assembly. Additionally, viability of fused tissue was confirmed on Day 4 by simultaneous live/dead fluorescent staining that showed no visible signs of decreased viability, suggesting that high viability was maintained throughout tissue fusion (Figure 3.3D). While other groups have shown that magnetic cellular spheroids, which incorporate MNPs using internalization or dispersed methods, can assemble magnetic cellular spheroids into tailored shapes and patterns, these studies often encounter adverse effects on cell viability and are therefore inapplicable for long-term tissue engineering strategies [8, 9]. These results provide proof of concept for magnetically manipulated
JMCSs to serve as building blocks in large three-dimensional tissue constructs using a variety of shapes and sizes.

Figure 3.3. JMCSs Aligned and Fused into Homogenous Tissues using Magnetic Force Assembly. (A-C) JMCSs were patterned and fused together into rings of varying sizes, ranging from 2 mm (A, 25 JMCSs, scale bar = 1000 µm) up to 10 mm (B,C 3,000 JMCSs each, scale bar = 10 mm). (D) Furthermore, viability of the fused structure was confirmed using simultaneous live/dead (green/red) fluorescent staining (scale bar = 1000 µm). (E,F) Finally, using various magnetic patterns, spheroids assembled onto custom patterns can fuse together over the course of days to form unified constructs, demonstrated by (E) a square and (F) Clemson University Tiger Paw. Images shown were allowed to fuse over the course of 4 days (scale bars: E = 5 mm, F = 10 mm).

The assembly of complex 3D tissues using cellular spheroids is heavily reliant upon efficient fusion of spheroids into a single tissue. To determine factors affecting JMCS fusion, spheroid fusion was analyzed by tracking the fusion of spheroids into controlled patterns using various JMCS and assembly parameters. When placed in
contact, cellular spheroids will fuse together, with the tissue contracting into a single tissue construct [6]. Twenty five individual JMCSs were magnetically assembled into a ring and their fusion was tracked. Images were taken daily and fusion was tracked by measuring changes in the inner and outer diameters of fused tissue rings. We varied the strength of the ring magnet to determine the effect of magnetic force on JMCS fusion to mediate magnetic cellular spheroid fusion, as JMCSs patterned using the weakest magnetic force (10% of maximum magnetic field) showed an increased rate of contraction of the inner diameter compared to other samples patterned with higher magnetic field strengths (Figure 3.4). All samples tested using 10% of the maximum magnetic field collapsed on themselves, leaving behind no inner diameter at late time points, results opposite of those seen using a high (100%) and medium (25%) magnetic fields. Rings fabricated using high and medium magnetic fields fused into cohesive rings while maintaining their desired final shape. All rings were magnetically assembled into symmetric rings on Day 0 after initial seeding. We hypothesized that rings patterned using 10% of the maximum magnetic field assembled into more asymmetric rings after 48 hours of fusion (with the magnet present) due to the necessity of sufficient magnetic force for effective magnetic patterning. Additionally, we analyzed the effect of spheroid MNP content on JMCS fusion. Results showed that similar to the strength of the magnetic field, lower MNP content lead to a statistically significant increase in rate of contraction (Figure 3.5) due to a decrease in the total magnetic field applied to the tissue with lower amounts of MNPs present. However, when varying magnetic forces (magnitude and spheroid MNP content), changes in outer diameter were not as significant.
as those seen for inner diameter measurements (Figure 3.6). Once factors affecting spheroid assembly and fusion into complex tissues using magnetic forces are understood, these parameters can be customized and applied for the assembly of a variety of engineered tissues types. The dramatic reduction in internal diameter of fused rings has been reported by other groups when analyzing fusion of cellular spheroids, while also showing some slight differences in fusion between different cell types [17]. However, to our knowledge, this is the first report describing the mediation of tissue contraction using magnetic forces, possibly due to the changes of surface angle contact between spheroids [6].
Figure 3.4. Effect of Magnetic Field Force on JMCS Fusion. (A,B) Results showed that the strength of the magnet used for JMCS fusion played a critical role in their fusion into the desired shape. JMCS patterning using a weak magnet (10% of maximum magnetic field) showed a greater rate of inner diameter contraction than those patterned using stronger magnetic fields. All samples using 10% of the maximum field collapsed on themselves. Compared to 100% control samples, JMCSs patterned with 10% magnetic field force demonstrated statistically significant differences at all time points after 84 hours (* p < 0.05). Scale bars = 1,000 µm.
Figure 3.5. Effect of MNP Content on JMCS Fusion. (A,B) The MNP content within JMCSs was varied to determine its effect on spheroid fusion. Statistical analysis showed significant differences (* p < 0.05) for ID contraction rates at 60, 84, and 108 hrs between the two MNP concentrations, showing that lower MNP content with JMCSs lead to a greater rate of contraction compared to their higher MNP counterparts. Scale bar = 1000 μm.
Figure 3.6. Factors Affecting JMCS Ring Fusion – Outer Diameter. Results showed that the strength of the magnet used for JMCS fusion does not significantly effect spheroid fusion regarding the OD of rings when varying (A) magnetic field force or (B) MNP content. Statistical analysis did show significant differences when using 25% magnetic field force compared to 100% samples at all time points after 84 hours (* p < 0.05).
3.3.2. Assembly of Complex Tissues

Next, we expanded on the assembly of complex tissues using JMCS to assemble a 3D vascular tissue construct. Compared to top-down tissue engineering approaches, directing tissue assembly with bottom-up approaches has become appealing due to control over construct spatial arrangement. As the bottom-up approach is intrinsically a modular technique, it allows for precise control over the tissue construct microenvironment and the ability to scale fabrication techniques [18, 19]. Results demonstrated the ability of individual JMSCs to fuse together into a complex 3D tissue whose assembly is driven by magnetic forces. Using magnetic forces and 2,000 to 4,000 JMCSs, spheroids were first magnetically patterned into a cellular strip, fused into a tissue strip (Figure 3.7 A,B), and wrapped around a tube with a removable magnetic within its lumen (Figure 3.7 C,D). Finally, the magnet within the lumen was removed, leaving behind a fused tube tissue composed solely of JMCSs (Figure 3.7 E,F). This method was applied to a variety of JMCS compositions, including varying cells numbers within JMCSs (Figure 3.7 G,H), cell types (Figure 3.7I), and even multicellular bilayered tubes using both SMCs and FBs (Figure 3.7J). The use of magnetic force manipulation and patterning allowed for the rapid assembly of a complex tube tissue composed of tailored cell, ECM, and MNP content using magnetic forces. The dimensions of the vascular tissue construct are controlled by the silicone tube diameter, cell strip length, and cell strip width. This approach allows for the assembly of a cell-based vascular tissue possessing a similar composition to native vessels by controlling JMCS’s cell, ECM, and MNP composition.
Figure 3.7. Assembly and Fabrication of Complex Tube Tissue. (A) Janus magnetic cellular spheroids were assembled on top of a magnet template, (B) fused into a strip after 4 days, magnet removed (C,D) wrapped around the tube using magnetic attraction, and (E,F) assembled as a fused tube after 6 additional days (10 days total). Additionally, this technique was be expanded for use with: (G,H) varying cell densities, (I) cell types (Fibroblasts, FBs), and (J) even bilayered multicellular tubes (SMC and FB, 0.24 mg/mL collagen I).
We next analyzed the role of time in mediating fusion of JMCSs into a complex vascular tissue construct (Figure 3.8). Bilayered vascular tissue constructs were assembled and histologically analyzed after four and ten days of assembly. Results showed that fusion time is critical in assembling complex tissues, as samples analyzed at day four still presented individual spheroids within the tissue core. Samples analyzed at day ten showed clear fusion of individual JMCSs into a single fused tissue. Kelm et al. showed that the application of fluid flow to the lumen of the fused tissue led to an increased fusion of assembled spheroids into a tissue tube after 14 days [20]. Here, we demonstrated that by applying magnetic forces during fusion, we can effectively control the fusion of JMCSs into a single fused tissue without fluid flow.

Figure 3.8. Fusion of Complex Tube Tissue. Bilayer tissue tubes were assembled using four thousand JMCSs (2,000 SMCs, 2,000 FBs) and revealed that fusion time is important for tissue assembly, as tissues at day four still presented individual JMCSs (scale bar = 5 mm, magnified = 500 µm).
Complex tissue tubes composed of multiple cell types were assembled to evaluate the potential for tailored JMCSs to assemble a functional vascular tissue construct. Using magnetic force assembly, thousands of JMCSs were assembled into a fused tissue tube 5 mm in diameter. As a proof of concept, a tissue tube mimicking both the spatial composition and content of a small diameter blood vessel was assembled. Tissue tubes were analyzed using histological staining to visualize the spatial localization of collagen and cells within the tissues. First, a unilayer tube composed of solely SMCs was assembled (Figure 3.9A). Next, a bilayer tube composed of an internal layer of SMCs and an external layer of FBs was fabricated using the same method (Figure 3.9B). Ultimately, a trilayer tube composed of an internal layer of endothelial cells, medial layer of SMCs, and external layer of FBs was assembled (Figure 3.9C). Cells were spatially oriented in this manner to mimic the tunica intima (endothelial cells), tunica media (SMCs) and tunica adventitia (FBs) of native blood vessels. Furthermore, analysis was performed to confirm control over the spatial location of cells within assembled tissues. Three separate trilayer tubes were assembled, with one cell type labeled within each tube. Results showed that the spatial location of different cell types could be tailored to mimic that orientation of native vascular tissues, confirmed by an external layer of FBs (Figure 3.9D), medial layer of SMCs (Figure 3.9E), and internal layer of ECs (Figure 3.10F). All tissue tubes were assembled using cellular spheroids containing 0.24 mg/mL collagen. Tissue tubes were assembled using high collagen spheroids in order to maintain a patent lumen due to the structural and mechanical properties provided by collagen. Other groups have demonstrated spatial control of multicellular vascular tissue
constructs, but require complex printing equipment for assembly [21]. Additionally, this approach to wrap a magnetic cell sheet around a magnetic mandrel has been previously utilized to assemble multicellular tissue tubes [22]. However, Ito et al. induced internalization of MNPs into cells prior to cell sheet assembly, and will likely encounter adverse effects related to MNP uptake in long-term applications [23, 24]. The presented strategy to assemble multicellular vascular tissue constructs demonstrates a simplistic and cheap approach to spatially control tissue engineered building blocks into tissue tubes. This strategy can be applied to any building blocks capable of magnetic force manipulation assembly. Furthermore, the use of JMCSs, which have been shown to reduce adverse effects on cell phenotype and viability, are appealing for tissue engineering strategies which require long-term interaction between MNPs and cells.

To further justify the use of high collagen spheroids for tissue tube assembly, the mechanical stability of tissue tubes was analyzed. Bilayer tissue tubes were assembled using both high (0.24 mg/mL) and low (0.017 mg/mL) collagen spheroids as previously described. Results showed that upon removal of the tissue tubes from the mandrel after day ten of assembly, low collagen tubes immediately collapsed (Figure 3.10). In contrast, high collagen tissue tubes remained open and patent upon removal from the mandrel. These high collagen tubes were self-retaining and capable of physical manipulation while low collagen tubes were incapable of physical handling. Therefore, due to the immediate collapse and inability for physical handling, low collagen tissue tubes were inadequate as a functional tissue engineered construct. These results suggest that in addition to mediating fusion, collagen content plays a critical role for structural
support when fabricating complex tissue structures composed of fused spheroids. An advantage of tissue engineering with spheroids is that fusion can occur within days, but the mechanical properties of the tissues tend to be inadequate [5]. Collagen provides a natural scaffold for the fusing tissue at its early stages, which allows the tissues to maintain structure and potentially be handled for introduction into post-processing techniques for maturation [2].
Figure 3.9. Assembly of Vascular Tissue Construct using JMCSs. Tissue tubes were successfully assembled using JMCSs to fabricate tubes composed of one, two and three different cell types to mimic the spatial orientation of cells within a blood vessel. (A) A single layer tissue tube composed of SMCs alone was assembled. (B) Next, a bilayered tissue tube composed of an internal layer of SMCs and an external layer of FBs was assembled. (C) Finally, a trilayered tissue tube composed of an internal layer of ECs, a medial layer of SMCs, and an external layer of FBs was assembled using magnetic force assembly. (D-F) To confirm the presence of three distinct cell types in different spatial locations, three separate trilayer tubes were assembled and analyzed (L = lumen). For each tube, one different cell type was labeled prior to tissue assembly (green). The whole tissue was stained for nuclei to visualize the location of other non-labeled cells and tissue tube boundaries. Results showed that three different cell types could be incorporated into a trilayer tube, demonstrating spatial control over the orientation of each cell type to mimic the native orientation found in vascular tissues. Results indicate that JMCSs can be
successfully fabricated into tissue tubes mediated by magnetic forces and incorporate multiple cell types with control over spatial alignment of cells and tissue composition. Scale bars: A-C = 5 mm, D-F = 500 µm

**Figure 3.10. Effect of Collagen Content on Tissue Tube Structural Support.** Bilayer tissue tubes were assembled with high (0.24 mg/mL) and low (0.017 mg/mL) collagen contents to determine the role of collagen in structural support. Results showed that collagen content plays a critical role in structural integrity, as low collagen tissue tubes collapsed upon removal from the mandrel template, while high collagen tissue tubes remained open and capable of physical handling.

3.4. Conclusions

We demonstrated that JMCSs can be used to successfully assemble and manipulate spheroids into controlled patterns and mediate their fusion over time, a strategy that can be used to assemble larger 3D tissue constructs. These results show that it is critical to control the magnetic forces to mediate fusion of tissues using magnetic cellular spheroids. The ultimate goal of this technology will be the assembly of larger tissue structures using magnetic force assembly, with the additional opportunity to use magnetic forces and manipulation for tissue construct maturation. While the ability to tailor the ECM and cellular content and composition of cellular spheroids makes them
applicable for tissue engineering, care must be taken when designing tissues in order to optimize tissue fusion based off the spheroid formulation used.

3.5. Works Cited


CHAPTER 4

BIological MagNETIC CELLULAR SPHERoIDS AS BUILDING BLOCKS FOR TISSUE ENGINEERING

4.1. Introduction

A variety of nanoparticles (NPs) including magnetic iron oxide NPs, gold NPs, carbon nanotubes, and polymeric NPs have been integrated with tissue engineering to provide in situ imaging, drug delivery, mechanical properties, and functionality [1, 2]. Nanotechnology can benefit tissue engineering due to its ability to control interactions at sub-cellular levels that are not possible using common tissue engineering techniques [3-5]. Particularly, magnetic nanoparticles (MNPs) have been integrated with tissue engineering applications for tissue patterning and maturation [6-9]. However, the direct and long-term interaction of MNPs with cells can induce adverse effects on cell viability, phenotype, and function, and therefore remain a critical concern. Commonly investigated MNPs include ferrites (cobalt [10], manganese [11], nickel [12]), manganites, as well as metals (Fe [13], Co, Ni [14]) and their alloys. However, before these ferrite NPs may be used in biomedical applications, their surfaces must be modified with polymers [15], gold, or silica [16] to improve or reduce their toxicity. Because of the complex chemistry inherent in such modifications, there is a critical need to investigate biological MNPs as an alternative to commonly used iron oxide MNPs which effectively reduce adverse effects on cells, thereby allowing for long-term use in tissue engineering applications.
Magnetoferritin is a potential biological MNP that can address the adverse cellular effects of common metallic MNPs. The primary role of ferritin, a natural protein in the body, is iron storage, with normal blood serum levels ranging from 10-200 ng/mL, and mean values of 103 ng/mL and 35.6 ng/mL for males and females, respectively [17]. In this work, we attempted to elucidate how to use magnetoferritin NPs to serve as a biological alternative to iron oxide MNPs. Specifically, we prepared magnetoferritin NPs with tailored loading and magnetic properties, analyzed its effects on cell viability, and demonstrated its ability to mediate tissue patterning via magnetic force assembly. The results demonstrate that magnetoferritin NPs have the potential to mitigate the cytotoxicity that currently prevents prolonged use of MNPs in tissue engineering applications.

4.2. Materials & Methods

An overview of experimental procedures, parameters, and objectives can be found in Table 4.1.
Table 4.1. Experimental Plan for Magnetoferritin Synthesis and Evaluation.
Experiments were performed to synthesize magnetoferritin NPs with tailored MNP content, analyze the interaction of magnetoferritin NPs with cellular spheroids based
histological examination to visualize spatial localization, quantification of internalization into cells, viability of cells within cellular spheroids, and the ability for magnetoferritin cellular spheroids to be utilized for magnetic force assembly in tissue engineering applications.

4.2.1. Materials

Apoferitin (equine spleen), Trimethylamine-N-oxide ((CH$_3$)$_3$N(O), 98%), AMPSO (C$_7$H$_{17}$NO$_5$S, 99%), ammonium (II) sulfate hexahydrate ((NH$_4$)$_2$Fe(SO$_4$)$_2$•6H$_2$O), potassium ferrocyanide (K$_4$Fe(CN)$_6$•3H$_2$O, 98.5-102%) were supplied by Sigma-Aldrich. Hydrochloric acid (HCl, 6 N) was supplied by Ricca Chemical Company. PrestoBlue Cell Viability Reagent and Collagen, Type I Bovine were supplied by Life Technologies. Commercial iron oxide MNPs were supplied by SkySpring Nanomaterials, Inc. (Fe$_3$O$_4$, 20 – 30 nm).

4.2.2. Statistical Analysis

All statistical analysis was performed using a two-tailed t-test with at least three repeats each. Statistical significance was set at p < 0.05. Error bars on graphs represent the standard deviation from the mean.

4.2.3. Magnetoferritin Synthesis

Magnetoferritin NPs were synthesized by gradually loading apoferritin with iron oxide NPs [18]. Apoferritin (0.44 M) was placed in a 3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonice acid (AMPSO) buffer solution (50 mM, pH 8.6) and heated to 65 °C. Aliquots of ferrous ammonium sulfate (Fe$^{2+}$, 0.1 M) and triemethylamine-N-oxide (Me$_3$NO, 0.07 M) were added dropwise to the reaction solution. Each addition of Fe(II) (0.612 µmol) was followed by a stoichiometric aliquot
of \( \text{Me}_3\text{NO} \) (3 Fe(II): 2 \( \text{Me}_3\text{NO} \), 0.612 µmol : 0.41 µmol) and stirred for 15 minutes before repeating the stepwise addition. Samples were dialyzed in water at 4 °C for two days prior to use.

4.2.4. Magnetoferritin NP Characterization

A bichinchoninic acid (BCA, Lambda Biotech, Inc.) assay was performed to quantify the amount of protein. Iron content within magnetoferritin NPs was quantified using an established technique to quantify iron content in solutions [19]. Briefly, NPs were first dissociated using 5 N HCl, followed by quantification of free iron within solutions using a Perl’s Prussian blue colorimetric technique. Additionally, magnetoferritin NPs were characterized using scanning transmission electron microscopy (STEM) and energy dispersive spectrometry (EDS) on a Hitachi 2000 STEM scope.

4.2.5. Cell Culture

Primary rat aortic smooth muscle cells (SMCs) were used for all cellular spheroid studies. Cells were cultured in monolayer cultures using Dulbeco’s Modified Eagle Medium:F-12 (ATCC, 1:1, DMEM:F-12) supplemented with 10% fetal bovine serum (Atlanta Biologics) and 1% penicillin-streptomycin-amphotericin (MediaTech, Inc.) at 37 °C and 5% of CO_2.

4.2.6. Cellular Spheroid Assembly

To assemble cellular spheroids, MNPs (magnetoferritin NPs or iron oxide NPs), collagen (Bovine, Type I, Life Technologies), and cells in cell culture media were dispensed using a modified hanging drop method. Unless otherwise noted, all spheroids were assembled using SMCs with MNPs, 20,000 cells per spheroid, and collagen.
Commercial iron oxide MNPs were used (Fe$_3$O$_4$, 20 – 30 nm, SkySpring Nanomaterials, Inc.).

4.2.7. Quantification of NP Uptake

First, cellular spheroids (10, Dispersed and Uptake) were dissociated. Dissociated samples were suspended in 1 mL fresh media and placed on a magnetic wash tool for five minutes. The supernatant was collected, and the remaining cells (magnetically attracted) suspended in 1 mL fresh media. The amounts of cells in the supernatant and magnet solution were quantified using a hemocytometer. Spheroids were collected and analyzed after three days of assembly in hanging drop.

4.2.8. Cellular Spheroid Viability

Magnetoferritin NP cellular spheroids were fabricated with 500 µg/mL magnetoferritin NPs and compared to control cellular spheroids without magnetoferritin NPs and spheroids with iron oxide MNPs. PrestoBlue cell viability assays were performed to quantify cell viability after spheroid dissociation (at least 3 repeats per sample).

4.2.9. Histology

Magnetoferritin cellular spheroids were processed and sectioned via standard paraffin sectioning techniques. Samples were dehydrated using ethanol and xylene prior to being embedded in paraffin. Sections 5 µm thick were stained with hematoxylin and eosin, and Lillie’s Technique for Turnbull's Blue Reaction (Poly Scientific).
4.2.10. Magnetic Patterning

Commercial ring magnets (SuperMagnetMan, 10 mm diameter), were secured to the bottom of glass chamber slides. Four hundred magnetic cellular spheroids were placed in the chamber and allowed to magnetically align. Tissue structures were allowed to fuse for four days prior to imaging, and magnet patterns were kept static throughout the four days and removed for imaging. Samples were imaged using a Nikon AZ100 multizoom microscope. Different concentrations of magnetoferritin NPs and iron oxide MNPs were used due to the magnetic attraction of cellular spheroids.

4.3. Results & Discussion

4.3.1. Magnetoferritin Synthesis & Characterization

By varying the number of cycles (from 10 to 70) performed during magnetoferritin NP synthesis, we were able to tailor the loading of iron oxide into equine spleen apoferritin protein shells. By increasing the number of synthesis cycles, we increased the iron oxide loading per ferritin shell from 810 to 3395 (iron oxide per protein, Figure 4.1A). Additionally, by varying the iron oxide content within magnetoferritin NPs, we were able to control the magnetic properties of these biological MNPs, as a higher loading content corresponded to stronger superparamagnetic properties with the application of an external magnetic field. In our subsequent analysis of our magnetic manipulation of magnetoferritin NPs, we determined that 70 cycle magnetoferritin NPs were subject to magnetic manipulation and attraction in contrast to unloaded apoferritin NPs (Figure 4.1A). Furthermore, magnetoferritin NPs were
characterized using scanning-transmission electron microscopy (STEM) and energy-dispersive spectroscopy (EDS, Figure 4.1B). Z-contrast was used for STEM imaging to highlight the iron oxide cores from their protein shell. Results confirmed that magnetoferritin NPs contained iron oxide, as indicated by the presence of both iron (red) and oxygen (blue) compared to apoferritin controls in EDS analysis. We used EDS to quantitatively confirm the iron content from magnetoferritin formulations, as demonstrated by an atomic percent increase of iron for 10 and 70 cycle magnetoferritin NPs (12.6% and 29.5%, respectively) compared to unloaded apoferritin (0.52%). The small amount of iron present in apoferritin samples was likely due to residual iron from the unloading of the native ferritin NPs. By controlling the loading of iron oxide into apoferritin shells and therefore the magnetic properties of magnetoferritin NPs, we demonstrated the ability to prepare tailored biological magnetic NPs.
Figure 4.1. Characterization of Magnetoferritin Nanoparticles. (A) The loading of iron oxide into magnetoferritin NPs was successfully controlled and resulted in magnetic NPs (70 cycle, compared to unloaded apoferritin protein NPs). Results confirmed control over loading content into magnetoferritin NPs based on reaction cycles performed. (B) Magnetoferritin NPs were analyzed using STEM and EDS to confirm the presence of an iron oxide core, as noted by the high expression of iron and oxygen in magnetoferritin NPs compared to apoferritin protein shells. Magnetoferritin NPs were characterized by an atomic weight percent increase in iron between 10 (12.6%) and 70 cycle (29.5%) magnetoferritin NPs, confirming an increased presence of iron oxide. Scale bars: apoferritin, 10 cycle = 600 nm, 70 cycle = 300 nm.
4.3.2. Magnetoferritin Cellular Spheroids

To determine the capacity for magnetoferritin NPs to serve as effective magnetic NPs for tissue engineering applications, magnetoferritin NPs were incorporated into cellular spheroids. Specifically, using rat aortic smooth muscle cells, magnetoferritin NPs (70 cycles) were incorporated into cellular spheroids using two techniques: uptake and dispersed (Figure 4.2A). Uptake cellular spheroids refer to spheroids composed of cells that have internalized magnetoferritin NPs. Dispersed cellular spheroids refer to a method of spheroid synthesis in which magnetoferritin NPs are dispersed throughout the extracellular space. Histological analysis was performed to analyze the dispersion of magnetoferritin NPs within cellular spheroids (Figure 4.2B). Samples were stained using hemotoxylin and eosin (H&E), and Lillie’s Turnbull Blue Reaction to show the presence of iron oxide within magnetoferritin NPs (iron = black). Magnetoferritin was visible in both stains, appearing red-orange in H&E, and black in Lillie’s Turnbull Blue Reaction. Results confirmed the dispersion of magnetoferritin NPs throughout the cellular spheroids using both spheroid formulations (uptake and dispersed) at magnetoferritin concentrations of 82 µg/mL. Furthermore, as the internalization of iron oxide MNPs into cells can induce cytotoxic effects, interaction between cells and MNPs should be minimized [20]. Compared to iron oxide MNPs, results showed that the percentage of cells internalizing magnetoferritin NPs with dispersed cellular spheroids was significantly lower (14%) than that of iron oxide MNPs (28%) (Figure 4.2C). Additionally, multiple magnetoferritin NP contents were histologically analyzed (Figure 4.3). These results
indicate that magnetoferritin NPs can be effectively incorporated within magnetic cellular spheroids with reduced internalization into cells.
Figure 4.2. Magnetoferritin Nanoparticles in Magnetic Cellular Spheroids. (A) Magnetoferritin NPs were incorporated into cellular spheroids using two methods: uptake and dispersed. Uptake spheroids contain cells that have internalized magnetoferritin NPs, while dispersed spheroids contain magnetoferritin NPs spatially distributed throughout the extracellular space. (B) Histological examination confirmed the assembly of cellular spheroids using both methods. Samples were stained with H&E and Lillie’s Technique for Turnbull’s Blue Reaction (Iron) to visualize the location of magnetoferritin NPs (Scale bar = 500 µm). (C) Using dispersed cellular spheroids, results showed that magnetoferritin NPs resulted in significantly lower cell uptake compared to iron oxide MNPs (* p < 0.05).
Figure 4.3. Tailored Magnetoferritin Content in Cellular Spheroids. Cellular spheroids containing various amounts of magnetoferritin were histologically examined using H&E and Lillie’s Technique for Turnbull’s Blue Reaction (Iron) to confirm control over magnetoferritin content (Scale bar = 500 µm). Results showed that magnetoferritin content can be tailored during spheroid fabrication, confirmed by an increase in iron content (black, Iron) as magnetoferritin concentrations were increased from 10 µg/mL up to 41 µg/mL.

To determine if magnetoferritin NPs induced cytotoxic effects on cells, we analyzed the cell viability of magnetoferritin spheroids. In our examination of both spheroid types composed of 500 µg/mL magnetoferritin, we confirmed that the presence of magnetoferritin NPs did not adversely affect cell viability compared to controls without MNPs (Figure 4.4A). Notably, we maintained a high cell viability using magnetoferritin NPs as compared to conventional metallic iron oxide MNPs (20-30 nm) up to one week. Using metallic iron oxide MNPs at concentrations of 50 µg/mL, Ho et al. demonstrated noticeable toxicity in patterned cellular spheroids after only 48 hours, thereby preventing potential use in tissue engineering applications that require a long-term interaction with biological systems to be clinically applicable [6]. Unlike iron oxide
MNPs, magnetoferritin NPs are composed of a protein shell, which makes them quite suitable for the long-term interaction with biological systems for tissue engineering applications. Therefore, magnetoferritin NPs can serve as an alternative biocompatible magnetic NP to iron oxide MNPs.

Finally, to demonstrate the application of magnetoferritin NPs for tissue engineering, we prepared fused tissue rings as a model using magnetic force assembly. We first confirmed the magnetic attraction of magnetoferritin cellular spheroids (Figure 4.4B), and then magnetically patterned and fused these dispersed magnetoferritin cellular spheroids (500 µg/mL) over a four day period into a tissue ring (Figure 4.4C). Samples were compared to magnetic cellular spheroids with iron oxide MNPs as a control (300 µg/mL). Fusion was confirmed by the lack of individual cellular spheroids after four days of patterning, which formed a single homogenous tissue void of gaps upon removal of the magnetic pattern. Based on these results, magnetoferritin can serve as a biological alternative to metallic MNPs for magnetic force assembly of tissues.
Figure 4.4. Magnetoferritin Cellular Spheroids for Tissue Engineering. (A) Magnetoferritin cellular spheroids were compared to control cellular spheroids without any magnetic NPs and iron oxide MNP cellular spheroids. Results confirmed that high viability was maintained up to one week compared to control spheroids without MNPs and magnetic cellular spheroids using iron oxide MNPs (* p < 0.05). (B) The ability of magnetoferritin cellular spheroids to magnetically attract to a permanent magnet was confirmed (magnet diameter = 10 mm). (C) Using magnetic force assembly, results showed a fused homogenous tissue, with magnetoferritin serving as a comparable alternative to iron oxide magnetic MNPs for patterning and fusion. Scale bar = 10 mm.
4.4. Conclusions

In conclusion, we have demonstrated that NPs can safely be integrated with tissue engineering applications using magnetoferritin NPs and serve as an alternative to iron oxide MNPs. The biological nature of magnetoferritin NPs has been shown to have the most potential in applications requiring long-term interaction with biological systems in that they exhibit no adverse effects on cell viability at concentrations much higher than that used with other metallic MNPs. In future research, we will expand upon our magnetoferritin NPs to fabricate complex three-dimensional tissue structures, multicellular tissues, study long-term biological response to cells, and test remote control of magnetic spheroids using magnetic forces.

4.5. Works Cited


CHAPTER 5

ACCELERATED IRON OXIDE NANOPARTICLE DEGRADATION MEDIATED BY POLYMER ENCAPSULATION WITHIN CELLULAR SPHEROIDS

5.1. Introduction

A variety of nanomaterials including gold nanoparticles, carbon nanotubes, polymeric nanoparticles, and magnetic iron oxide nanoparticles have been used in various tissue engineering strategies involving imaging, tissue maturation and integration, and drug delivery [1-9]. Magnetic nanoparticles (MNPs) are now being increasingly used in biomedical applications [10-20], with some FDA approved iron oxide MNP formulations used to treat iron deficiency (Feraheme) or as MRI contrast agents (Feridex) [21-23]. MNPs have been investigated in tissue engineering applications for in vivo cell tracking [24-29], in vivo monitoring of transplanted tissues [30-32], cell and tissue patterning [11, 13, 15], and tissue maturation [33]. However, the prolonged presence of MNPs can induce adverse effects in cells, causing cell toxicity, and changes in both cell phenotype and cell mobility [13, 34, 35]. Though the surface functionalization or coating of MNPs in oleates [36, 37], dextran [23, 38], or polymers [31, 39] can improve MNP biocompatibility, they do not control the MNP degradation. Ideally, MNPs will remain stable for a sufficient time to accomplish their desired task, and then rapidly degrade once their task is completed.

A variety of different chemicals, primarily organic acids, have been investigated to accelerate the degradation of iron oxide MNPs [40-44]. In these experiments, after the MNPs accomplished their desired task, the accelerated MNP degradation decreased their
interaction with cells. Here, we report the precise control of iron oxide MNP degradation via MNP encapsulation within biodegradable polymers for tissue engineering applications. Iron oxide MNPs encapsulated within polymeric nanoparticles are a biodegradable alternative to non-coated MNPs to remove MNP *in situ* in that they obviate potential toxicities. By accelerating MNP degradation, they may have broad use in medical devices, drug delivery, and bioimaging agents composed of iron oxides.

5.2. Materials & Methods

An overview of experimental procedures, parameters, and objectives can be found in Table 5.1.
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<th>Experiment</th>
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<td>•Determine effect of PolyMNPs on cell viability within magnetic cellular spheroids</td>
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<td>PolyMNP Cellular Spheroids for Magnetic</td>
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<td>•Analyze the capacity of PolyMNPs to be utilized for magnetic force assembly in tissue engineering applications</td>
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Table 5.1. Experimental Plan for PolyMNP Analysis. Experiments were performed to analyze the ability to tailor the encapsulation of MNPs inside polymeric NPs, determine the effect of polymer composition and content on MNP degradation, determine the effect of PolyMNPs on cell viability within magnetic cellular spheroids, and to utilize PolyMNP cellular spheroids for magnetic force assembly. Multiple parameters were varied within each experiment as noted.
5.2.1. Materials

Commercial MNPs (Fe$_3$O$_4$, 20 – 30 nm) were supplied by SkySpring Nanomaterials, Inc. D,l lactide (C$_6$H$_8$O$_4$, PURASORB DL) was supplied by Purac Biomaterials. Glycolide (C$_4$H$_4$O$_4$, >99%), tin(II) 2-ethylhexanoate ([CH$_3$(CH$_2$)$_3$CH(C$_2$H$_5$)CO$_2$]$_2$Sn, ~95%), sodium sulfate (Na$_2$SO$_4$, >99%), anhydrous magnesium sulfate (MgSO$_4$, >99.5%), anhydrous toluene (C$_6$H$_5$CH$_3$, 99.8%), methanol (CH$_3$OH, >99.9%), chloroform (CHCl$_3$, >99.8%), and potassium ferrocyanide (K$_4$Fe(CN)$_6$•3H$_2$O, 98.5-102.5%) were supplied by Sigma-Aldrich. Methoxy-poly(ethylene glycol) was supplied by JenKem Technology USA (M-PEG-OH, Mw 5000). Acetonitrile (C$_2$H$_3$N, 99.9%) and Tetrahydrofuran (C$_4$H$_8$O, 99.9%) were supplied by Fisher Scientific. Hydrochloric acid (HCl, 6 N) was supplied by Ricca Chemical Company. PrestoBlue Cell Viability Reagent and Collagen, Type I Bovine were supplied by Life Technologies.

5.2.2. Statistical Analysis

All statistical analysis was performed using a two-tailed t-test with at least three repeats each. Statistical significance was set at p < 0.05. Error bars on graphs represent the standard deviation from the mean.

5.2.3. Polymer Synthesis & Characterization

Block copolymers of PLA-PEG or PLGA-PEG were synthesized via ring opening polymerization using methoxy-poly(ethylene glycol) as the initiator and tin(II) 2-ethylhexanoate as the catalyst [45]. PLGA composition was controlled by varying the ratio of d,l lactide and glycolide. Reagents were dissolved by stirring in 120 °C toluene
under N\textsubscript{2} gas and reflux. Tin(II) 2-ethylhexanoate was added and reaction vessel was stirred at 120 °C for 24 hours. The next day, polymer product was washed in chloroform/water, dried over MgSO\textsubscript{4}, and precipitated in cold methanol. NMR was performed with a Bruker Avance 300. \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}, δ): 7.26 (s, CDCl\textsubscript{3}), 5.17 (q, -C(=O)-CH(CH\textsubscript{3})-), 4.82 (d, -C(=O)-CH\textsubscript{2}-O-), 3.65 (s, -CH\textsubscript{2}CH\textsubscript{2}-O-), 1.59 (d, -CH(CH\textsubscript{3})-). ATR FT-IR was performed with a Thermo-Nicolet Magna 550 equipped with a Thermo-SpectraTech Foundation series Endurance Diamond ATR. IR: 2881 cm\textsuperscript{-1} (-CH\textsubscript{2}CH\textsubscript{2}-O-), 1745 cm\textsuperscript{-1} (C=O). TGA was performed on a TA Instruments Hi-Res TGA 2950 thermogravimetric analyzer under nitrogen from 25 °C to 600 °C at 20 °C/min.

5.2.4. Polymer Degradation

Polymers were dissolved in acetonitrile (ACN, 50 mg/mL) and dispensed into a non-treated 96-well plate (125 µL, 6.25 mg). Plates were left overnight under a chemical hood to evaporate ACN, leaving behind a polymer film. Wells were filled with PBS and incubated at 37 °C until their respective time point. At each time point, PBS was removed and samples were washed three times with Hyclone Molecular Biology Grade Water (Fisher Scientific). Samples were dissolved in ACN and collected to be dried via lyophilization. The Polymer Molecular Weight was determined through gel permeation chromatography (GPC) on a Waters 1525 Binary HPLC pump with a Waters 2414 refractive index detector. A Shodex KF-804L column (8.0 x 300 (mm) ID x Length) and Shodex KF guard column were used for separation. The mobile phase was tetrahydrofuran (THF) and polymers were prepared by dissolving in THF at a concentration of 1 mg/mL and filtering through a 0.2 μm PTFE syringe filter (VWR
International). Flow rate was set at 1 mL/min and polystyrene standards (Poly-Sciences) were used to quantify molecular weight using a third-order fit calibration curve. The MW of samples at each time point was expressed as a percent initial.

5.2.5. PolyMNP Encapsulation

MNPs were encapsulated into polymeric nanoparticles via solvent evaporation [46]. Briefly, equal volumes of polymer (5 mg/mL) and iron oxide were dispersed in ACN and combined with Hyclone Molecular Biology Grade Water at a 1:2 ratio. Solutions were stirred for two hours, then washed in centrifugal filter units (100 kD MWCO, 3,500 RPM for 8 minutes) twice with water, and once with PBS. Final solutions were suspended in cell culture media or PBS depending on the application.

5.2.6. Cell Culture

Primary rat aortic smooth muscle cells (SMCs) were used for all cellular spheroid studies. Cells were cultured in monolayer cultures using Dulbecco’s Modified Eagle Medium:F-12 (ATCC, 1:1, DMEM:F-12) supplemented with 10% fetal bovine serum (Atlanta Biologics) and 1% penicillin-streptomycin-amphotericin (MediaTech, Inc.) at 37 °C and 5% of CO₂.

5.2.7. PolyMNPs in Cellular Spheroids

To assemble JMCSs, MNPs (PolyMNP or iron oxide NPs), collagen (Bovine, Type I, Life Technologies), and cells in cell culture media were dispensed using a modified hanging drop method. Unless otherwise noted, all spheroids were assembled using SMCs with MNPs, 20,000 cells per spheroid, and collagen. Commercial iron oxide MNPs were used (Fe₃O₄, 20 – 30 nm, SkySpring Nanomaterials, Inc.).
5.2.8. Quantification of PolyMNP Degradation

Iron content within cellular spheroids was quantified using an established technique to quantify that content in solutions [47]. Briefly, MNPs were first dissociated using 5 N HCl, followed by quantification of free iron within solutions using a Perl’s Prussian blue colorimetric technique. Magnetic cellular spheroids containing PolyMNPs (0.13 – 0.2 mg/mL) were fabricated and incubated in non-treated 96-well plates in cell culture media with media changes every other day. At each time point, cellular spheroids were transferred to 1 mL of sterile PBS in a sterile 1.5 mL microcentrifuge tube to wash samples. Spheroids were then transferred into 100 µL fresh PBS in a non-treated 96-well plate. 100 µL of 5 N HCl was added to each well and incubated at 37 °C for 72 hours to dissociate MNPs. Next, 100 µL of 5% potassium ferrocyanide was added to each well and incubated for 15 minutes at room temperature, with absorbance measurements recorded at 630 nm for each well.

5.2.9. Cellular Spheroid Viability

PolyMNP cellular spheroids were fabricated with 0.5 mg/mL PolyMNP and compared to control cellular spheroids without PolyMNPs. PrestoBlue cell viability assays were performed to quantify cell viability (at least 3 repeats per sample). Spheroids were dissociated via incubation with collagenase at 37 °C (Collagenase Type IV, Life Technologies). Cellular spheroids were then physically dissociated and allowed to adhere overnight on a tissue culture treated 12-well plate.
5.2.10. Histology

PolyMNP cellular spheroids were processed and sectioned via standard paraffin sectioning techniques. Samples were dehydrated using ethanol and xylene prior to being embedded in paraffin. Sections 5 µm thick were stained with hematoxylin and eosin, and Lillie’s Technique for Turnbulls Blue Reaction (Poly Scientific).

5.2.11. Magnetic Patterning & Fusion

Axially magnetized ring magnets (SuperMagnetMan, 2 mm OD, 1 mm ID, vendor calculated pull force = 0.16 lbs.) were commercially purchased and secured to the bottom of glass chamber slides containing coverglass bottoms. Twenty-five magnetic cellular spheroids (0.14 mg/mL PolyMNP) were placed in the chamber and allowed to magnetically align. Samples were incubated at 37 °C with 5% CO₂ for 48 hours. Magnets were removed after 48 hours and samples imaged using a Nikon AZ100 multizoom microscope.

5.3. Results & Discussion

5.3.1. Polymer Encapsulated MNPs (PolyMNPs)

MNPs were loaded into polymeric NPs using a solvent evaporation technique with two different polymers, poly(lactide)-poly(ethylene glycol) (PLA-PEG) and poly(lactide-co-glycolide)-poly(ethylene glycol) (PLGA-PEG), and purified to eliminate free polymer and free MNPs to form PolyMNPs (Figure 5.1A). The generation of polymer degradation byproducts, lactic and glycolic acid, created an acidic microenvironment within the polymeric NPs [48]. Furthermore, the dissolution of iron
oxide by acids occurred in three primary steps: the adsorption of organic ligands on the iron oxide surface, then non-reductive dissolution, and finally reductive dissolution (Figure 5.1B) [44]. FT-IR analysis showed that MNPs were encapsulated into polymeric NPs with a carbonyl stretch peak at 1745 cm\(^{-1}\) corresponding to the ester bond in PLA (Figure 5.2A). Thermogravimetric analysis (TGA) was used to quantify the relative weight percent of polymer composition of PolyMNP formulations. By varying the MNP:polymer weight ratios at 1:1.85, 1:5, and 1:10, we achieved 20, 25, and 33 wt% polymer, respectively (Figure 5.2B). The degradation kinetics for the two polymers used for encapsulation, PLA-PEG and PLGA(75/25)-PEG, were obtained via gel permeation chromatography (GPC). Results indicated a variance in polymer degradation based upon polymer composition (Figure 5.3). PLGA(75/25)-PEG molecular weight (Mw) decreased faster compared to PLA-PEG, losing roughly 18% of its initial Mw after 2 weeks in PBS at 37 °C due to its copolymer composition and higher hydrophilicity [49, 50]. PLA-PEG lost approximately 3% of its initial Mw under the same conditions. By tailoring the degradation kinetics of polymeric NPs, it is possible to control the MNP degradation rate due to the increased content of oligomer residues from polymer degradation at the interface of iron oxide MNPs and the polymer coating. Polymeric microparticles and nanoparticles have been observed to degrade over time, forming a local acidic microenvironment generated from degradation byproducts including lactic and glycolic acid. The formation of a local acidic core within polymeric NPs has been shown [48, 51]. Furthermore, Miller et al. confirmed that lactic acid (0.1 M), a degradation byproduct of PLA, led to dissociation of iron oxide after 100 hours at room temperature,
demonstrating roughly 80% degradation at pH 3.5 but only 10% degradation at pH 5.5 [43]. However, other studies have shown that the use of lactic acid (0.12-0.16 M) to expose cells to pH 5.7 or below induced cell toxicity [52]. Therefore, the use of polymeric NPs to encapsulate MNPs permits the control of a local acidic microenvironment within the degrading polymer NP that will in turn accelerate encapsulated MNP degradation.
Figure 5.1. Iron Oxide Degradation. (A) MNPs were encapsulated within polymeric NPs prepared using conventional nanoprecipitation method. Two different polymers, PLA-PEG and PLGA(75/25)-PEG, were used to prepare PolyMNPs. As both degrade at different rates, they generate a differential content of polymer degradation byproducts such as lactic acid and glycolic acid. (B) A schematic of the dissolution of iron oxide by acids, specifically by polymer degradation byproducts lactic acid and glycolic acid, which occurs in three primary steps: adsorption of organic ligands on the iron oxide surface, non-reductive dissolution, and finally reductive dissolution [44].
Figure 5.2. PolyMNP Characterization. (A) Using PLA-PEG NPs, polymer encapsulation of PolyMNPs was tested for three MNP:polymer ratios: 1:1.85, 1:5, and 1:10. FT-IR showed the presence of PLA-PEG for all three formulations of the PolyMNP assembly compared to the non-encapsulated MNP control. (B) Thermogravimetric analysis was performed to determine the polymer:MNP content. Results showed that the lowest MNP:polymer ratio (1:1.85) correlated to the highest MNP content (80 wt% MNP), while the highest MNP:polymer ratio (1:10) corresponded to the lowest MNP content (63 wt% MNP).
5.3. Polymer Degradation. Polymer degradation analysis was performed for both polymers used for PolyMNP encapsulation. Of the two polymers examined, PLGA(75/25)-PEG degraded the fastest (green), losing 18% of its initial molecular weight after two weeks in PBS at 37 °C. PLA-PEG (blue) demonstrated a slower degradation rate, losing 3% of its initial molecular weight under identical conditions. Statistical analysis indicated significant differences between the two polymers after three days (* p < 0.05).

5.3.2. PolyMNP Degradation

The encapsulation of MNPs within polymeric NPs accelerated MNP degradation, and MNP degradation was controlled by varying the polymer composition. Using 1:5 PolyMNP conditions, the degradation of MNPs encapsulated into both PLGA(75/25)-PEG and PLA-PEG NPs was measured after incubating PolyMNPs within cellular spheroids [47]. Briefly, PolyMNPs cellular spheroids were first treated with 5 N HCl to dissociate MNPs within cellular spheroids, followed by quantification of the free iron within the HCl solutions using a Perl’s Prussian blue colorimetric technique. PolyMNP degradation was compared to non-encapsulated MNP degradation over the course of 28 days. PLGA(75/25)-PEG PolyMNPs demonstrated a 21% degradation of the initial iron
content, while PLA-PEG PolyMNPs showed only a 12% degradation and non-coated MNPs only a 7% degradation over the same time period (Figure 5.4A). These results, which correlate with the polymer degradation rates (Figure 5.3), showed that the PLGA(75/25)-PEG degraded more quickly than PLA-PEG. Polymer degradation is in part controlled by the differential hydrophilicity in the polymer composition. Increasing the polymer degradation rate is expected to more rapidly increase the content of the acidic monomer and oligomer byproducts at the MNP interface [48, 49, 53]. Conversely, a slower degradation of polymers is expected to slowly degrade MNPs due to a lower content of acidic monomer and oligomer byproducts. The degradation of PolyMNPs was further qualitatively confirmed by histological staining of cellular spheroids over the course of 40 days using H&E and Lillie’s Turnbull Reaction to highlight iron oxide MNPs (Figure 5.4B, PLA-PEG PolyMNPs Figure 5.5). Results showed that similar to non-encapsulated MNPs, PolyMNPs dissociate over time into smaller aggregates of MNPs, a dissociation that corresponds to a degradation of the MNPs shown in Figure 5.3A [41]. PolyMNP degradation studies were also performed using PLGA(50/50)-PEG PolyMNPs in cellular spheroids (Figure 5.6), as well as using all three polymers in PBS (Figure 5.7A). A primary concern of using MNPs for tissue engineering is their long-term presence in human tissues, with often adverse effects. A safe method that can accelerate this MNP degradation, however, is expected to limit the interaction of MNPs with the biological environment, which is critical in reducing cytotoxicity. The presented results confirm that the polymer composition of PolyMNPs can control the degradation rate of MNPs in physiological conditions. This accelerated degradation, compared to
non-encapsulated MNPs, makes PolyMNPs most appealing for use as MNPs in biomedical applications that necessitate limited interaction with the biological environment.
Figure 5.4. Effect of Polymer Composition on PolyMNP Degradation. (A) Two polymers possessing different degradation rates were used to prepare PolyMNPs: PLA-PEG and PLGA(75/25)-PEG. A Perl’s reagent (potassium ferrocyanide) assay was used to measure the degradation of PolyMNPs encapsulated into both polymer nanoparticles after incubation of PolyMNPs within cellular spheroids [47]. An analysis of that degradation within cellular spheroids showed that polymer degradation rates mediate MNP degradation. PLGA(75/25)-PEG PolyMNPs, the fastest degrading polymer of the two formulations tested, demonstrated the most accelerated MNP degradation compared to PLA-PEG PolyMNPs and non-encapsulated MNP controls over the course of 28 days. Statistical analysis showed significant differences (* p < 0.05) after two weeks compared to raw MNPs. (B) Degradation of PolyMNPs was also qualitatively analyzed using histological staining (H&E and Lillie’s Turnbull for Iron) over the course of 40 days.
Results showed that PolyMNPs dissociate into smaller aggregates over time, correlating with quantitative MNP degradation analysis (Scale bar = 500 µm).

Figure 5.5. PLA-PEG PolyMNP Degradation in Cellular Spheroids. Histological analysis of cellular spheroids containing PLA-PEG PolyMNPS confirmed the degradation of MNPs over the course of 40 days. Degradation was demonstrated by dissociation of MNPs into smaller aggregates over time. Results were confirmed with H&E and Lillie’s Turnbull Reaction to visualize iron oxide dissociation. Scale bar = 500 µm.
Figure 5.6. PLGA(50/50)-PEG PolyMNP Degradation. (A) Polymer degradation analysis confirmed that PLGA(50/50)-PEG polymer degraded with time, losing 5% of its initial MW after 14 days in PBS at 37 °C. (B) PolyMNPS were assembled using PLGA(50/50)-PEG NPs and a MNP degradation analysis within cellular spheroids showed that these PolyMNPs resulted in a 16% reduction in initial iron content after 28 days.
Figure 5.7. PolyMNP Degradation in PBS. The degradation of MNPs within PolyMNPs was also analyzed in PBS at 37 °C. (A) The effect of polymer composition on PolyMNP degradation was assessed using three different polymers: PLA-PEG, PLGA(75/25)-PEG, and PLGA(50/50)-PEG. PolyMNPs were assembled using 1:5 MNP:polymer reaction conditions. Results showed that similar to PolyMNP degradation in cellular spheroids, PLGA(75/25)-PEG PolyMNPs resulted in the highest MNP degradation, losing 11% of its initial iron content after 28 days. PLA-PEG and PLGA(50/50)-PEG PolyMNPs resulted in 3% and 5% loss of initial iron content after 28 days, respectively, while raw MNPs lost only 1% of their initial iron content. Statistical analysis showed significant differences for all samples compared to raw MNPs (* p < 0.05) at all time points except PLGA(50/50)-PEG at day 7. (B) The effect of
encapsulation efficiency on PolyMNP degradation was also analyzed in PBS. Similar to PolyMNP degradation in cellular spheroids, PLA-PEG PolyMNs were used for all reaction conditions. Results showed that similar to degradation in cellular spheroids, PolyMNs assembled with the highest encapsulation efficiency (1:10) resulted in the most accelerated MNP degradation, demonstrating 64% loss of their initial iron content compared to raw MNPs which lost only 16% of their initial iron content. PolyMNs assembled using 1:5 and 1:1.85 reaction conditions resulted in losses of 62% and 33% of their initial iron content, respectively. Statistical analysis showed significant differences for all samples compared to raw MNPs (* p < 0.05) at all time points except 1:1.85 at two and three weeks. However, a statistically significant difference was seen again for 1:1.85 samples at four weeks.

The effect of polymer content to PolyMNP degradation was subsequently analyzed. By varying the relative content of polymer encapsulating MNPs within polymeric NPs, we controlled the rate of MNP degradation. By increasing the content of polymer, we created less MNPs within the polymeric NPs and accelerated MNP degradation (Figure 5.8). As mentioned earlier, TGA results showed that by varying MNP:polymer weight ratios during synthesis (Figure 5.2B), we controlled the content of polymer encapsulation of MNPs. Having a higher content of polymer encapsulating MNPs within NPs is expected to increase MNP degradation because of the higher content of monomer and oligomer degradation byproducts [54]. Therefore, the results suggest that the increased presence of degradation byproducts accelerates MNP degradation [48]. Specifically, in experiments using cellular spheroids with PolyMNs prepared with 1:10 (lowest loading, 33 wt% polymer) and 1:1.85 (highest loading, 20 wt% polymer), we observed a 74% and a 10% reduction of their initial iron content after 28 days, respectively. These results indicate that MNP degradation can be tailored by both polymer composition and content. PolyMNP degradation using various encapsulation
efficiencies was also performed in PBS, demonstrating the same trend shown in cellular spheroids (Figure 5.7B).

![PolyMNP Degradation in Spheroids Encapsulation Efficiency](image)

**Figure 5.8. Effect of Polymer Content on PolyMNP Degradation.** Using PLA-PEG PolyMNPs, a degradation analysis was performed to determine the effect of polymer content on MNP degradation. The degradation of PolyMNPs composed of 1:1.85 and 1:10 MNP:polymer ratios was analyzed over the course of 28 days in cellular spheroids using a Perl’s reagent assay [47]. Results showed that PolyMNPs with the highest polymer content (1:10) correlated to the most accelerated degradation over the course of 28 days, because of the increase in monomer and oligomer degradation byproducts within polymeric nanoparticles. Statistical analysis indicated significant differences (* p < 0.05) between the two samples after two weeks.

5.3.3. PolyMNPs in Cellular Spheroids

Since magnetic cellular spheroids are used as components in tissue engineering applications to assemble complex tissues via magnetic force assembly [55-57], which causes prolonged MNP and cell interactions, we analyzed PolyMNP cytotoxicity.
PolyMNPs assembled using PLA-PEG and PLGA(75/25)-PEG polymeric NPs were incorporated into magnetic cellular spheroids and analyzed for cytotoxicity over a period of two weeks. PolyMNPs, 0.5 mg/mL, maintained high viability until the end of that time for both PolyMNP formulations, compared to control spheroids without MNPs (Figure 5.9A). Furthermore, compared to spheroids composed of cells which internalized raw MNPs, PLGA(75/25)-PEG PolyMNP spheroids maintained a high viability compared to control spheroids without MNPs up to one week (Figure 5.10). Raw MNP spheroids showed viability below 20%, compared to control spheroids. Viability studies were also performed at lower PolyMNP concentrations of 0.15 mg/mL (Figure 5.11), demonstrating no adverse effects on cellular spheroids viability up to two weeks. Additionally, magnetic cellular spheroids with PolyMNPs were used to magnetically pattern and assemble fused tissues (Figure 5.9B). Tissue rings, 2 mm in diameter, were successfully patterned and assembled using both PolyMNP formulations, with an equivalent fusion compared to non-encapsulated MNPs. Magnetic cellular spheroids containing PolyMNPs were magnetically patterned and fused together over the course of 48 hours, with fused tissue observed after the removal of the magnetic template. These results demonstrated that PolyMNPs maintain high cellular viability and promoted fusion, suggesting that PolyMNPs are a biodegradable alternative to non-encapsulated MNPs for tissue engineering applications.
Figure 5.9. PolyMNPs Integrated Within Cellular Spheroids. (A) The effect of PolyMNPs on cellular spheroid viability was analyzed to determine the suitability of PolyMNPs for prolonged cellular interaction. Results showed that PolyMNPs (0.5 mg/mL) can be incorporated into cellular spheroids and maintain high viability compared to MNP-free controls for up to two weeks. Statistical analysis showed significant differences (* p < 0.05) compared to control spheroids without MNPs for PLA-PEG samples at one and two weeks, but in favor of PolyMNP spheroids (normalized ratio greater than 100%). PLGA(75/25)-PEG spheroids showed significant differences at all time points, with viability increasing compared to controls at one and two weeks. (B) Furthermore, PolyMNP-containing cellular spheroids were magnetically patterned and assembled into fused tissue rings. Twenty-five individual PolyMNP spheroids were patterned and fused over the course of 48 hours, at which point the magnet pattern was removed and the samples imaged. Results showed a fused tissue construct, confirming that PolyMNPs can be used to assemble fused tissue rings with equivalent fusion compared to non-encapsulated MNP controls. Scale bar = 1,000 µm.
Figure 5.10. PolyMNP Spheroid Viability. Compared to control cellular spheroids without MNPs, PLGA(75/25)-PEG PolyMNPs maintained high viability over the course of one week. Cellular spheroids assembled using cells which had internalized raw MNPs (non-coated) showed significantly reduced viability (* p < 0.05), demonstrating viability below 20% compared to MNP-free controls.

Figure 5.11. Effect of PolyMNPs on Cellular Spheroid Viability. The effect of PolyMNPs on cellular spheroid viability was analyzed to determine the suitability of PolyMNPs for prolonged cellular interaction. Studies were performed at lower PolyMNP concentrations (than those previously reported). Results showed that PolyMNPs (0.15 mg/mL) can be incorporated into cellular spheroids and maintain high viability compared to MNP-free controls for up to two weeks. Statistically significant differences were seen at one week (* p < 0.05), but in favor of PolyMNP spheroids (normalized ratio greater than 100).
5.4. Conclusions

Results showed that the degradation of MNPs can be controlled by the polymeric microenvironment. It was further demonstrated that the degradation rate of MNPs within cellular spheroids can be controlled by varying the polymer composition and content of PolyMNPs. Additionally, high cell viability was maintained in magnetic cellular spheroids up to two weeks, which is a most desirable characteristic of these spheroids for use as constituents for tissue engineering applications requiring prolonged interaction between MNPs and cells. Finally, PolyMNP spheroids were magnetically patterned to promote fusion into homogenous tissues, confirming that magnetic force assembly can be used to pattern PolyMNPs. These results demonstrated that the polymeric microenvironment could be used to precisely control the degradation of MNPs in physiological conditions, which will limit the interaction of MNPs with cells. Consequently, this process is invaluable in creating methods to use MNPs in medical applications, in drug delivery, and as bioimaging agents composed of iron oxide. Future research will entail the use of different polymers for PolyMNP encapsulation, an analysis of the molecular changes at the interface between MNPs and polymers, and a study of the degradation of macrostructures.

5.5. Works Cited


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CHAPTER 6
CONCLUSIONS

Nanotechnology can improve upon current biomedical strategies by mimicking the native biological structure at a sub-cellular level. While MNPs have been incorporated into tissue engineering strategies to provide in situ imaging, drug delivery, and tissue patterning, the direct and prolonged interaction of MNPs with cells can induce adverse effects on cell viability, phenotype, and function, and therefore remain a critical concern. Consequently, methods to reduce any adverse effects and interaction of MNPs with cells will benefit tissue engineering strategies requiring prolonged interaction. Key challenges encountered when integrating nanotechnology, particularly MNPs, into tissue engineering strategies include: 1) internalization of NPs into cells, 2) mediating sub-cellular interactions during tissue assembly, 3) complex chemical modifications to improve biocompatibility of NPs, and 4) prolonged interaction between NPs and cells. The presented results demonstrate that a variety of methods can be used to improve upon the use of MNPs within tissue engineering. Using cellular spheroids as a model, results showed that MNPs can be safely and effectively incorporated into tissue engineering strategies.

6.1. Janus Magnetic Cellular Spheroids to Reduce MNP Internalization

Cellular spheroids have been investigated as tissue engineered building blocks due to their ability to mimic the native 3D cellular and ECM structure of tissues [1-4]. Magnetic forces can be utilized to pattern and manipulate cellular spheroids for assembly of complex tissues, with a variety of methods used to incorporate MNPs within cellular
spheroids [5-11]. Common methods include internalization into cells [12-15], dispersion throughout the ECM space [5, 12, 16], or conjugation to the cellular membrane [7, 11, 17]. While these have proven effective in short term studies, adverse effects regarding cell viability and phenotype are encountered due to internalization of MNPs within cells [5, 6, 15, 17, 18]. We developed a novel method to incorporate MNPs within cellular spheroids possessing two segregated domains, one composed of cells and the other of extracellular MNPs. We hypothesized that this separation of MNPs and cells would limit the interaction of MNPs and cells, thereby avoiding adverse effects on cell viability and phenotype. Compared to cellular spheroids assembled using cells that have internalized MNPs (Uptake spheroids), JMCSs maintain high long-term viability and stable phenotype expression compared to MNP-free controls. Viability was maintained using a variety of ECM compositions, contents, cell types, and cell densities, confirming that this technique can be applied to assemble tailored tissues for desired applications. Other groups have shown adverse effects on spheroid viability at iron oxide MNP concentrations of 50 µg/mL [6] and adverse effects on cell phenotype at 240 µg/mL [19]. However, the presented results used iron oxide MNPs at 300 µg/mL for all cell viability and phenotype studies (unless otherwise noted), a concentration higher than that reported by other groups while avoiding any adverse effects. Furthermore, while other groups have dispersed MNPs throughout the entire extracellular space of cellular spheroids [5, 12, 16], the presented Janus technique localizes MNPs into a segregated extracellular region, which further limits the interaction between MNPs and cells. The rationale for studying this is to understand the effect of MNPs and magnetic cellular spheroid
composition on cell viability and phenotype expression. These results confirm that the method by which MNPS are incorporated into cellular spheroids is critical for effective and safe tissue engineering strategies. Furthermore, the presented Janus technique can serve as an effective method to incorporate MNPs into cellular spheroids while avoiding adverse effects common to MNP internalization.

6.2. Promoting and Mediating Spheroid Fusion via Magnetic Force Assembly

Although cellular spheroids fuse together when placed within contact of each other, methods to align and maintain spheroids in a set location in 3D are needed before complex tissues can be fabricated. Methods to pattern and align spheroids include: direct seeding into a mold [20, 21], printing into a hydrogel or biopaper [2, 22, 23], or surface
patterning to promote adhesion [24-27]. Although these are effective, they require complex material fabrication or modification to be functional, therefore limiting their use in large-scale tissue engineering strategies. The use of magnetic forces to manipulate and align cellular spheroids can be utilized for this patterning and expanded into tissue engineering. Magnetic force assembly and patterning is appealing for tissue engineering as it allows for action at a distance and can be easily patterned and controlled using solid or custom magnetic fields. Once we demonstrated that MNPs could be incorporated into cellular spheroids while avoiding adverse effects related to their internalization, we utilized JMCSs to assemble and manipulate cellular spheroids into controlled patterns and complex tissues. We hypothesized that JMCSs could be utilized to assemble tailored complex tissues using magnetic force assembly. Results showed that using magnetic force manipulation, JMCSs could be assembled into a variety of controlled patterns regardless of shape or size. Next, results demonstrated that magnetic forces can be used to mediate fusion of cellular spheroids into more complex tissues, confirming that it is critical to control magnetic forces used during tissue assembly. Using a vascular tissue construct as a proof of concept, thousands of JMCSs were successfully assembled into tailored complex tissues. Other groups have demonstrated assembly of vascular tissue constructs using printed bioink [22] or cellular spheroids [21]. Tissue tube assembly with printed bioink showed variations in tissue fusion throughout the constructs [22]. The presented results confirm that magnetic forces mediate and accelerate spheroid fusion. Kelm et al. showed that the application of fluid flow to the lumen of the fused tissue led to an increased fusion of assembled spheroids into a tissue tube after 14 days [21]. Here,
we demonstrated that by applying magnetic forces during fusion, we can effectively control the fusion of JMCSs into a single fused tissue without fluid flow. The rationale for these studies is that magnetic forces can be used to align JMCSs into a controlled pattern and drive their fusion by maintaining these JMCS in close contact with one another, thereby accelerating and enhancing spheroid fusion compared to unpatterned assembly. Results highlight that a variety of factors mediate spheroid fusion into complex tissues, and care must be taken to optimize these parameters based on desired outcomes.

**Figure 6.2. Promoting Spheroid Fusion using Magnetic Force Assembly.** Results showed that magnetic forces could be used to promote the fusion of JMCSs into complex tissues, with magnetic force properties mediating their fusion into homogenous tissues.

6.3. **Biological Magnetoferritin NPs to Improve MNP Biocompatibility**

A variety of MNPs have been investigated for imaging, drug delivery, and tissue patterning techniques, but commonly investigated biomedical MNPs are limited due to adverse effects on cells [5, 6, 15, 17, 18]. These metallic MNPs often involve complex surface modification to improve or reduce cytotoxic effects [28, 29]. Therefore, magnetoferritin, a biological MNP derived from ferritin, was investigated as a biological alternative to inorganic iron oxide MNPs. We hypothesized that magnetoferritin NPs
could be synthesized with tailored magnetic properties and serve as a biologically based MNP for tissue engineering applications. Results showed that we could tailor the loading and magnetic properties of magnetoferritin NPs, and safely incorporate them into cellular spheroids without any adverse effects on cell viability. Furthermore, magnetoferritin cellular spheroids were magnetically patterned and fused into controlled patterns using magnetic force manipulation. Other groups have utilized magnetoferritin NPs for tumor targeting [30] and cell sorting [31]. The presented results demonstrate the use of magnetoferritin NPs into tissue engineering applications. Furthermore, while others have shown toxic effects of iron oxide MNPs at 50 µg/mL [6], the presented results show that spheroids assembled using cells which had internalized magnetoferritin NPs at 500 µg/mL showed no adverse effects on cell viability up to one week. The rationale for these studies is that inorganic MNPs often require complex chemistry to improve or reduce cytotoxic effect on cells, and that the biological nature of magnetoferritin NPs will improve upon MNP biocompatibility in tissue engineering applications. Results demonstrated that magnetoferritin NPs can serve as a biological alternative to inorganic iron oxide MNPs, mitigating the cytotoxicity that prevents the use of MNPs in tissue engineering applications.
Figure 6.3. Tailored Magnetic Properties of Biological Magnetoferritin NPs. Results showed that magnetic properties of magnetoferritin NPs could be tailored and improve on the biocompatibility of common iron oxide MNPs. Magnetoferritin NPs were incorporated into magnetic cellular spheroids with no adverse effects on cell viability, and were capable of magnetic force assembly for tissue engineering applications.

6.4. Accelerated MNP Degradation via Polymer Encapsulation

The prolonged presence of MNPs can induce adverse effects in cells, causing cell toxicity, and changes in both cell phenotype and cell mobility [6, 14, 19]. Though the surface functionalization or coating of MNPs in oleates [32, 33], dextran [34, 35], or polymers [36, 37] can improve MNP biocompatibility, they do not control the MNP degradation. Ideally, MNPs will remain stable for a sufficient time to accomplish their desired task, and then rapidly degrade once their task is completed. Therefore, we developed a method to accelerate the degradation of iron oxide MNPs via encapsulation within polymeric NPs. By encapsulating MNPs within polymeric NPs, we hypothesized that MNP degradation could be mediated by the local acidic microenvironment generated by polymeric NP degradation. Results showed that PolyMNP degradation could be tailored based on polymer content and composition. PolyMNPs were assembled using multiple biodegradable polymers, with results confirming control over MNP loading.
within polymeric NPs. To confirm their capacity for tissue engineering applications, PolyMNPs were incorporated into cellular spheroids and maintained high viability up to two weeks. Furthermore, PolyMNP cellular spheroids were patterned and fused into controlled shapes using magnetic force assembly. Groups have demonstrated that surface functionalization or coating can enhance MNP biocompatibility and functionality by improving cell viability, uptake, or biodistribution [37], but these techniques often involve complex chemistry and do not necessarily accelerate the removal of MNPs from the body. The presented technique offers a simplistic method to encapsulate MNPs within a biocompatible polymer that actively accelerates the degradation of MNPs. This technique requires no chemical modifications to MNPs and can be expanded to a variety of different NPs or polymers compositions for various applications. Furthermore, although studies have shown that lactic acid can accelerate MNP degradation at an acidic pH (pH 5.5 and below) [38], the pH required for degradation with these acids has been confirmed to induce toxic effects on cells (pH 5.7 or below) [39]. The presented results utilize the capacity of NPs to control the pH of the local microenvironment and accelerate MNP degradation via polymer degradation byproducts [40]. The rationale for these studies is to limit the interaction between MNPs and cells that is common to tissue engineering applications by accelerating the degradation of MNPs. These results demonstrated that the polymeric microenvironment could be used to control the degradation of MNPs in physiological conditions. This technique can be applied to strategies beyond tissue engineering to limit the interaction of MNPs with the biological environment.
Figure 6.4. Accelerated MNP Degradation. The degradation of MNPs was accelerated by encapsulation within polymeric NPs. Results showed that PolyMNPs could be assembled with tailored polymer content and composition, with MNP degradation dependent on polymer degradation rate and MNP loading.

6.5. Summary

The presented results highlight multiple strategies which can improve upon the biocompatibility of MNPs in tissue engineering applications. Results emphasize that limiting the internalization of MNPs within cells is critical to avoiding adverse effects on cell viability and phenotype. A variety of strategies including spatial segregation of MNPs away from cells, use of biologically-based MNPs, or decreasing the amount of interaction between MNPs and cells via accelerated degradation can be applied to common tissue engineering strategies to assemble complex tissues. While common tissue
engineering strategies which utilize internalization of MNPs within cells are effective in short-term, their prolonged interaction with cells limits their use in the development of functional engineered tissues capable of long-term use. Using a small diameter vascular construct as a platform, we have developed a broad range of nanomaterial strategies for tissue engineering which improve upon current MNP techniques.

6.6 Works Cited


CHAPTER 7

RECOMMENDATIONS

Based on the literature review and the limits of the presented results, a summary of future recommendations includes:

7.1. Analyses and Development of Methods to Induce JMCS Elastin Synthesis

While the ECM is composed of a variety of biomolecules, collagen and elastin provide critical structural and mechanical support for tissues [1, 2]. Additionally, the ability of cellular spheroids to secrete their own ECM makes them appealing for tissue engineering applications [3]. Preliminary results showed that the addition of MNPs to JMCSs promoted collagen synthesis within cellular spheroids compared to MNP-free controls. Next, it would be interesting to study the effect of MNPs on JMCS elastin synthesis, or develop methods to induce elastin synthesis within JMCSs. While MNPs alone may not induce elastin synthesis, treatment of JMCSs with chemical or growth factors may induce or accelerate its synthesis. A variety of chemical and growth factors including TGF-β1 [4], aldosterone [5], insulin [6], hyaluronic acid [7], and retinoic acid [8] have been shown to accelerate or induce elastin synthesis and should therefore be investigated with JMCSs.
7.2. Effect of JMCS Composition on Spheroid Fusion

Tissue printing techniques have been applied to assemble tailored complex tissues, but these tissues often encounter issues with incomplete or inconsistent fusion throughout [9-12]. Results showed that the magnetic force properties used during magnetic force assembly were critical in mediating JMCS fusion. Therefore, future studies should be performed to determine the effect of other spheroid parameters, such as ECM composition, content, and cell density, to determine their effect on JMCS fusion.

7.3. Assembly & Maturation of Various Tissues using JMCSs and Magnetic Force Assembly

Companies, such as Organovo, have utilized cellular spheroids to assemble biomimetic engineered tissues for use in therapeutic screening and disease modeling technologies, including the fabrication of a 3D liver tissue model. Our results confirmed that JMCSs could be assembled with a variety of ECM and cellular compositions and contents while avoiding adverse effects on cell viability. Therefore, JMCSs can be tailored to assemble a variety of tissues types based on target tissue native ECM and cellular compositions. The ability to control magnetic patterning will allow for the assembly of multicellular tissues with precise control over the spatial location of various JMCS types. Furthermore, MNPs distributed throughout assembled tissues could be utilized to induce tissue maturation using mechanical stretching via magnetic force manipulation. A variety of tissues, including skeletal muscle, cardiac, bladder, respiratory, digestive, and vascular tissues, require constant mechanical stretching within
the body to function. However, diseases and conditions such as atherosclerosis, muscular dystrophy, aneurysms, and pulmonary fibrosis interfere with a tissue’s natural ability for mechanical stretching, eventually leading to tissue failure. These diseases and conditions highlight the critical requirement for constant mechanical stretching in native tissues. Once engineered tissue constructs have been assembled, they often require additional treatment to induce maturation and improve mechanical properties to more closely mimic native tissues [13]. Mechanical stretching can be applied to improve the mechanical properties of engineered vessels [14-17], and is often applied using custom bioreactors or chemical treatments with histamine, potassium, noradrenaline, or serotonin [18, 19]. However, these strategies often involve long fabrication times (months), and are therefore cost and time consuming [20]. Magnetic force maturation has shown success in engineered bone constructs, inducing desired differentiation and ECM production with short treatment times (1-3 weeks) [21, 22]. Furthermore, unlike fluid flow or chemical maturation techniques which affect areas only in direct contact, magnetic maturation offers the ability to manipulate the tissue construct throughout, affecting all cells.

7.4. Improved Magnetoferritin NP Magnetic Properties

Though a magnetic NP may possess some magnetic properties, this does not necessarily correlate to application in magnetic force assembly. A magnetic NP must possess sufficient magnetic properties to be capable of magnetic attraction and manipulation within a functional magnetic field range. Studies have shown that aggregates of magnetoferritin NPs possess increased magnetic characteristics compared
to dispersed samples [23]. Our results showed that magnetoferritin NP magnetic properties could be tailored based on loading. Therefore, work should be performed to analyze other ways to increase the magnetic properties of magnetoferritin NPs via aggregation. The use of crosslinking would provide control over the amount of aggregation, with the additional benefit of being able to remove crosslinking via various treatment techniques (chemical, heat, pH, etc.).

7.5. Additional Studies on Magnetoferritin NPs

Studies utilizing iron oxide MNPs demonstrated the capacity for JMCSs to assemble complex 3D tissue using magnetic force assembly. Additionally, results showed that magnetoferritin cellular spheroids could be magnetically patterned and fused into a simple tissue ring. This work should be expanded to use magnetoferritin cellular spheroids for the assembly of complex and multicellular 3D tissues using magnetic force assembly, similar to previous studies done using iron oxide JMCSs to assemble a small diameter vascular tissue construct.

A key concern with MNPs is prolonged interaction with cells, demonstrating adverse effects on cell viability and phenotype [24-28]. Results analyzed the effects of magnetoferritin NPs on cell viability up to two weeks, but this should be expanded up to at least seven weeks similar to iron oxide JMCSs. Most tissue engineering applications require prolonged interaction between cells and MNPs on the scale of weeks, making long-term studies a key concern for effective application. Furthermore, studies should be performed to analyze the effect of magnetoferritin NPs on phenotype expression.
7.6. Additional Studies on PolyMNPs

Polymer degradation is mediated by a variety of factors including polymer molecular weight and composition [29, 30], as well as environmental factors such as heat and pH [31, 32]. Additionally, the degradation of different types of polymers will release various degradation byproducts which in turn have various effects on the surrounding microenvironment [33]. Results showed that three different biocompatible polymers composed of PLA, PLGA, and PEG could be utilized to assemble PolyMNPs and induce accelerated MNP degradation. It would be interesting to assemble other PolyMNPs composed of other polymeric components to determine the overall ability to control MNP degradation via polymer selection.

The degradation of MNPs occurs via chelation of surface iron [34]. Results showed that encapsulation of MNPs within polymeric NPs accelerates degradation, and we proposed a mechanism by which this occurs. Therefore, studies should be performed to analyze what is actually occurring at the surface of MNPs within PolyMNPs during degradation to determine various parameters that can be tailored to further accelerate degradation. This could lead to optimization of PolyMNP systems which could further accelerate MNP degradation.

As mentioned prior, the degradation of polymeric NPs and MNPs is dependent on a variety of local and environmental factors, with studies showing differences between the in vivo and in vitro rates of polymer degradation [35]. Studies confirmed that MNP degradation was accelerated in vitro in both PBS and cellular spheroids. Therefore, these
studies should be expanded to \textit{in vivo} degradation to ensure that MNP degradation is still accelerated using PolyMNPs compared to raw MNPs.

7.7. \textit{Works Cited}


CHAPTER 8
ACCOMPLISHMENTS
PUBLICATIONS

Original Contributions


- **Mattix, B.** Poole, J., Reese, L., Visconti, R., Simionescu, D., Simionescu, A., Alexis, F. “Spatial Control of Magnetic Nanoparticles Integrated with Cellular Spheroids as Tissue Engineered Building Blocks.” 2012. (submitted)


Reviews & Book Chapters


**Patents & Disclosures**


**CONFERENCE ORAL PRESENTATIONS**


**CONFERENCE POSTER PRESENTATIONS**


• **Mattix, B.**, Poole, J., Casco, M., Visconti, R., Simionescu, D., Simionescu, A., Alexis, F. “Spatial Control of Magnetic Nanoparticles Integrated with Cellular Spheroids as Tissue Engineered Building Blocks.” Biomedical Engineering Society 2012 Fall Symposium. New Orleans, LA.

• **Mattix, B.**, Poole, J., Casco, M., Visconti, R., Simionescu, D., Simionescu, A., Alexis, F. “Spatial Control of Magnetic Nanoparticles in Cellular Spheroids as Tissue Engineered Building Blocks.” Biomedical Engineering Society 2012 Annual Meeting. Atlanta, GA.


**AWARDS**

• Department Seminar Award. 1st place award for outstanding performance in scientific oral communication, 2012.