Microneedle Arrays for Injection Seeding of Tissue Engineered Scaffolds

Katelyn Michelle Rye
Clemson University

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MICRONEEDLE ARRAYS FOR INJECTION SEEDING OF TISSUE ENGINEERED SCAFFOLDS

A Thesis
Presented to
the Graduate School of
Clemson University

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

by
Katelyn Michelle Rye
December 2014

Accepted by:
Dr. Dan Simionescu, Committee Chair
Dr. Agneta Simionescu
Dr. Martine LaBerge
ABSTRACT

Tissue engineered constructs have proven to possess comparable mechanical and physical characteristics to autologous tissues making them suitable replacements for diseased anatomy when autologous tissue transplants are unavailable. However, difficulty in reseeding the constructs with cells prevents the tissue-engineered scaffolds from becoming an adequate, equivalent replacement for diseased tissues when compared to autologous transplants. Cells adhere to the outer surfaces of scaffolds but are incapable of penetrating the constructs to provide complete cellularization. If cells were able to populate the inner layers of the tissue, then they could likely diffuse into the intimal and external layers of the construct to create a fully colonized scaffold. We propose the use of microneedles to inject cells into the inner layers of scaffolds to evenly repopulate entire constructs with cells.

With recent advancements in microfabrication, arrays of microneedles, less than a millimeter in height, can be fabricated simply and cost effectively. These microneedle arrays can be attached to a syringe to allow injection of cells into the interior of the scaffold. Unlike traditional hypodermic needles, these microneedle arrays will allow for accurate and customizable penetration depth as well as a customizable distance between adjacent needles. With this microtechnology, anatomically accurate distances between neighboring cells can be achieved, an option that has never been previously available.

To validate the use of microneedles for bulk cell seeding, the group injected cells into both decellularized carotid arteries and decellularized aortic cusps using three
different arrays of microneedles. We then determined the overall cellular density in the tissue using histological methods. Mechanical damage was quantified through mechanical testing.
DEDICATION

I would like to dedicate this thesis to my family. Their continual love and support has allowed me to accomplish all of my goals and aspirations. I am lucky to have been blessed with such strong role models as parents; they have always encouraged me to put forth my best effort and remain optimistic. For their guidance, I am eternally grateful. Although we have been hundreds of miles apart for almost five years now, I have always felt my family’s encouragement and knew that they were there if I ever needed anything.

Clemson would not have been the same without the friends that I have made along the way. These past years have been the best years of my life because of the people that I have been able to share my life with.
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My gratitude is extended to my committee members: Dr. Martine LaBerge, Dr. Agneta Simionescu, and Dr. Dan Simionescu. Their wisdom and encouragement made the success of this project possible.

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CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Tissue Scaffolds

Tissue scaffolds can be produced from polymeric derivatives or decellularized xenogeneic tissues. While polymeric scaffolds are inherently nonantigenic structures and, therefore, do not pose the risk of eliciting an immune response, they do not exhibit the same mechanical or structural characteristics as native tissues. As an alternative to polymeric scaffolds, xenogeneic tissues can be decellularized to create collagenous scaffolds that are architecturally analogous to native tissues. Decellularization eliminates the possibility of an immune response, while also preserving proteins and signaling pathways present in the extracellular matrices of autologous tissues.

1.1.1 Blood Vessel Anatomy and Physiology

Blood vessels can be classified as veins, arteries or capillaries. Each vessel serves as a transport route for the delivery of blood and nutrients to organs and tissues and an export route for the removal of metabolic wastes. Arteries carry blood away from the heart and withstand higher pressures than veins. These pressures generate a consistent mechanical stress on the arteries.

Arteries are composed of three distinct tissue layers, the intima, media and adventitia, as shown below in Figure 1. The three layers differ in thickness and composition depending on their location in the body and the corresponding distance from the heart.
The tunica intima is a single, continuous layer of endothelial cells that lines the lumen of the vessel. The endothelial cells are bound together by tight junctions and gap junctions. The tightly bound endothelial layer creates a non-thrombogenic surface that prevents clotting and allows laminar blood flow (Ross, 2010). If this layer is damaged, a thrombus will form and cause a cascade of pathological events to occur.

The tunica media is composed mainly of smooth muscle cells that provide the contractile force of the artery. The smooth muscle cells secrete the collagen, elastin, and other proteins that make up the extracellular matrix of the media (Ross, 2010). As blood flows through the artery, the smooth muscle cells constrict or relax allowing the artery to dilate or constrict. Vasodilation and vasoconstriction control blood volume as well as allow blood pressure to be controlled and regulated. The blood vessel must withstand constant, physiological pressures ranging from 80mmHg to 120mmHg (Ross 2010).
The outermost layer, the adventitia, is primarily composed of connective tissue and fibroblasts. The adventitia prevents the artery from stretching past its physiological limits during vasodilation (Ross, 2010). The adventitia contains a network of blood vessels called the vaso vasorum that provide nutrients and remove waste from the inner layers of large, conducting arteries.

There are three different types of arteries: muscular, elastic, and arterioles; each differing in function and composition. As you move further away from the heart, the arteries decrease in size. The carotid artery is an elastic artery, with a thick tunica intima, thick media, and a thin adventitia. The carotid artery is one of the most commonly diseased areas of vasculature.

1.1.2 Blood Vessel Pathology

Cardiovascular disease, including coronary artery and peripheral vascular disease, is the leading cause of mortality in western countries and often requires vascular reconstructive surgery (Mayo Clinic). The limited supply of suitable small-diameter vascular grafts has led to the development of tissue engineered blood vessel substitutes. Damage to blood vessels is most commonly due to a process called atherosclerosis.
Atherosclerosis is defined as the buildup of plaque inside of an artery (National Institute of Health, 2014). Plaque is a deposit of fat, cholesterol, and calcium bound together by a fibrous matrix. The etiology of atherosclerosis is unknown, but studies have shown that its prevalence increases with smoking, poor diet, aging, and a past family history of heart disease. The process begins when diseased T-cells and monocytes leave the blood circulation and migrate into the artery wall. In the arterial wall, the cells transform into foam cells that are essentially cells that digest large amounts of cholesterol and fats. In time, the smooth muscle cells in the media move into the intimal space and accumulate there, forming the atherosclerotic plaque (Merck Manual, 2014).

Over time the plaque continues to grow blocking the lumen of the vessel, and thereby reducing blood flow. As the size of the plaque bolus increases, it loses stability. Eventually the unstable plaque may rupture and form a blood clot that occludes the artery. Debris of the ruptured plaque can travel through the bloodstream and become lodged in another blood vessel, sometimes cumulating in a stroke (American Heart Association).
1.1.3 Aortic Cusp Anatomy and Physiology

The heart beats about 2.5 billion times in a 70-year lifespan, making it the most mechanically stressed organ in the body. (Cleveland Clinic, 2014). The heart consists of four chambers; the top two atria receive blood while the bottom two ventricles pump blood. The four valves of the heart function together to control the direction and flow of blood. Each valve is composed of either two or three leaflets that are connected to a ring called the annulus. The anatomy of the heart is displayed in Figure 4 below, and the anatomy of the aortic leaflet is shown in Figure 5.

![Figure 4: Heart anatomy.](Mayo Clinic, 2014)

![Figure 5: Aortic valve anatomy.](Cross section of a cusp, showing the three layers present (Vesely, 1998)).

The leaflet is composed of three distinct layers: the fibrosa, spongiosa and ventricularis. The fibrosa is the top layer of the leaflet composed of elastin and circumferentially aligned collagen. The fibrosa maintains the durability and stiffness of the valve, allowing it to withstand pressures up to 80mmHg (Vesely, 2004). The middle layer, the spongiosa, contains glycoaminoglycans and loosely packed collagen. This
layer absorbs the shock of the changing pressures by allowing the ventricularis and fibrosa layers to move against each other (Schoen, 2005). The ventricularis is the bottom layer of the leaflet composed of radially aligned elastin. This layer responds to the pressure changes in the heart during the valve cycle.

Since the aortic valve is the most stressed valve in the heart, it is correspondingly the valve most prone to disease and is most commonly replaced (Guyton and Hall, 2006).

1.1.4 Aortic Cusp Pathology

The high stress placed onto the aortic valve sometimes culminates in valvular damage such as microtears and calcium deposits. Calcium is continually circulating in the blood stream, and microtears due to wear create crevices for the accumulation of calcium deposits. When calcium builds up in the valve, the leaflets become fibrotic producing a narrowed valve opening (Cleveland Clinic). Calcium buildup increases with high blood pressure and aging.

Congenital defects can also have detrimental effects on the aortic valve. Congenital defects include improper valve size, malformed leaflets, or an irregularity in which the leaflets are attached. In the specific case of bicuspid aortic valve disease, the patient is born with two leaflets instead of three, leading to a stenotic or leaky valve.
Figure 6: Aortic valve stenosis. The diseased aortic valve cannot open or close properly when the leaflets are calcified or stenotic. This results in regurgitant flow and leakage. (Edwards Lifesciences, 2014)

Although bicuspid aortic valve disease is most common, the patient can also have a unicuspid valve or quadricuspid valve; all of which result in a narrowing or leaky valve over time (Mayo Clinic).

Infective endocarditis and rheumatic fever are two common infections that lead to valve failure. Rheumatic fever, often caused by untreated strep throat infection, causes the leaflets to become inflamed or to stick together and become scarred, rigid or thickened (Cleveland Clinic). Over time rheumatic fever can lead to the formation of scar tissue, creating an uneven surface on which calcium deposits can accumulate (Mayo Clinic). Endocarditis is a bacterial infection in the blood stream that culminates on the cusps. The bacterium causes growths on the valves and eventually leads to valve stenosis.

Aortic valve stenosis is commonly a first step in an ongoing cascade that leads to other serious cardiovascular pathologies. For example, when the aortic valve narrows,
the left ventricle must work harder to pump blood. To compensate for the extra work, the left ventricle may thicken and enlarge, leading to an overall weakening of the heart. A weak heart may lead to angina, heart failure, arrhythmias and cardiac arrest (Mayo Clinic).

1.1.5 Partial Conclusion

About 600,000 people die of heart disease in the United States every year—that’s 1 in every 4 deaths (Murphy, 2010). The commonality of cardiovascular disease leads to a high demand for solutions. In many cases of artery or valve failure, there is no easy fix and the entire structure must be replaced. The tissue engineering of cardiovascular tissues can create fully functional, integratable organ substitutes to meet this high demand.

1.2 Microneedles

Hypodermic needles are the standard of use for interdermal drug delivery. Hypodermic needles are about 25mm in length and range from 1-11mm in diameter. Although these needles are highly effective for the delivery drugs or small molecules to the patient, they have their drawbacks. Hypodermic needles cannot be safely used by the patient at home and often require trained assistance for use. In addition, the needles are painful. The length of the needle allows the tip to penetrate the dermis and disrupt nerve endings thereby causing pain and discomfort. By decreasing the needle height to a micron level, the effectiveness of the needle as an injection device can be preserved while the associated pain can be eliminated. Accordingly, a shorter needle eliminates the need
for trained assistance. With recent advances in microfabrication technology, microneedles have now become a reality for drug delivery.

1.2.1 Microneedle Classification

Several different configurations of microneedles exist for subcutaneous small-molecule delivery. These needles are classified as solid, particle-coated, dissolving, or hollow. Each needle type is fabricated differently to meet its intended use.

![Microneedle Classification Diagram](image)

**Figure 7: Mechanism of delivery and classification of microneedles.** (Kim, 2012). (a) The needles are inserted, and pressed flush against the tissue (b) Solid: The needles are removed and drugs applied topically diffuse into the pores. Coated: drugs diffuse off of the surface of the needle. Dissolving: the needle degrades releasing the drug suspension. Hollow: drugs are expelled through the needle bores into the tissue.

Solid microneedles are used for pretreatment of the skin or tissue. The solid needles can be painlessly inserted into the skin and then removed to leave a micron sized pore in the tissue. A drug can then be applied topically to the porated surface and absorbed via diffusion down into the tissue. Solid microneedles have been sold for cosmetic use since 1999 when Horst Liebl founded Dermaroller, a brand selling a roller made of microneedles for home-use to treat scars, stretch marks, and wrinkles.
Solid microneedles are the easiest to fabricate out of all the various microneedle classes.

Coated microneedles use a slow-release mechanism in which a water-soluble drug formulation diffuses off of the needle exterior into the surrounding tissue. After an appropriate amount of time, the needle can be removed and the drug remains in the tissue. The amount of drug released to the patient depends on the surface area of the needle as well as the method of coating. Single layers of a drug can be coated onto the needle using a dipping or spraying method; or multiple layers can be bound to the needle by alternatively charging the drug positively or negatively each time a new layer is added (Saurer, 2010).

Dissolving microneedles are fabricated to fully degrade upon placement in the tissue. These needles are commonly constructed out of water-soluble, biodegradable substances that encase the drug. Over time, the entirety of the needle will dissolve and the drug will diffuse locally throughout the tissue. The use of these needles is limited to the formulation and properties of the drug (Kim, 2012).

Hollow microneedles attach to a syringe and operate identically to hypodermic needles. Essentially, hollow microneedles are shorter micro-versions (100um-2000um) of hypodermic needles. The miniature dimensions of these needles make them much more complicated to fabricate than their longer, traditional counterparts.

Preliminary studies were conducted in our lab using solid microneedles to determine if poration by microneedles will increase cell infiltration. Results (not included) showed a dramatic increase in cellular retention in dry tissues but no infiltration...
in wet tissues. We theorized that surface tension prevented the cells from infiltrating the tissue and displacing water molecules. Based off of these results, we hypothesize that the use of hollow microneedles will allow the use of both hydrated and dehydrated scaffolds—the hollow microneedles will use the pressure of the syringe to force the cells into the interior layers of the scaffolds regardless of the presence of liquid molecules.

1.2.2 Hollow Microneedle Fabrication

Microneedles have been successfully fabricated from silicon, metal, polymer, glass and ceramics using a combination of photolithography, silicon etching, laser cutting, metal electroplating, metal electropolishing, micromolding, as well as concepts from microelectromechanical system engineering. The basis for deciding which process to use depends on the geometry of the needle and the material.

The most common practice in fabricating hollow microneedles is substrate etching using microelectromechanical systems (MEMS), yet many other methods using silicon, metal and polymer molds have been successful as well (McAllister, 2003). MEMS evolved from the technology in semiconductor device fabrication, using microscaled techniques such as wet etching, dry etching, and electro-discharge machining. Each of these processes involves the deposition of material layers, patterning by photolithography, and then etching to produce the required shapes. An illustration of fabrication using MEMS was created in MEMS Pro software (softMEMS), a CAD design program for the development and testing of MEMS-based products.
Figure 8: Process of creating hollow microneedles using MEMS (a) Silicon substrate (b) Deposition of SiO$_2$ (c) Backside etching of SiO$_2$ (d) Deep reactive ion etching (e) Deposition of Si$_3$N$_4$ (f) Pattern transfer for front side (g) Isotropic etching for needle shaft (h) Removal of SiO$_2$ and Si$_3$N$_4$ (i) Final shape (Amin, 2013)

Figure 8 above depicts the fabrication of a 200um, single, hollow, out-of-plane microneedle with MEMS. First a solid block of the desired material is chosen and cut to the proper height. Next, mask material is added to each side using low-pressure chemical vapor deposition (LPCVD). Photoresist is then deployed over the mask material using a lithography process patterning. Following this, the needle bore is formed using deep reactive ion etching. LCPCVD is used again to coat and protect the needle bore. Next, photoresist in deposited onto the length of the device that will not form the needle to protect it from future etching steps. The needle is then created using isotropic underetching. Finally, any remaining remnants of the oxide and nitride layers are removed (Amin, 2013).
1.2.3 Potential Applications to Tissue Engineering

As far back as the 1970’s, scientists began experimenting with low cost microneedle arrays for drug delivery (Gerstel, 1976). Only with recent advances in micromachining technologies has the creation of microneedles become affordable, effective, and reproducible. Several studies have already been completed to validate the use of microneedles for the delivery of small molecules.

Microneedle injection has proven to be an effective method for the administration of vaccinations, insulin, and polymeric microspheres (van der Maaden, 2014; Gupta, 2008; Wang, 2006). A study comparing the injection of the polio vaccine into rats using a hypodermic needle versus a single microneedle showed comparable IgG responses and antibody induction. This demonstrates that the microneedle was as equally effective as the hypodermic needle at delivering the vaccine to the rat (van der Maaden, 2014). The polio vaccine is usually delivered using a 23-25-gauge needle (OD 0.51mm-0.64mm), which is large enough to easily accommodate a 0.02mm cell (Immunization Action Coalition, 2014). The successful delivery of a vaccine serves as a predicate for the successful delivery of cells.

The flow rate of the injected solution through the microneedle has also been investigated. Studies conducted on human cadavers measured flow rates of 130uL/hour through individual needles. This rate could be further increased with a slight retraction of the needle after insertion to create a pocket for the fluid to fill (Martanto, 2006). This is evidence to support that a low viscosity cell suspension should be able to flow into an animal tissue.
Potential damage to the skin after repeated injection has also been a common cause for concern with microneedle arrays. Post-insertion and retraction, microholes with diameters ranging from 100-300um in diameter remain in the skin. These holes disappear within 10-20 minutes in in-vivo models and after 30 minutes there is no evidence of the injection (Wang, 2006). This demonstrates that damage to the living tissue is minute and temporary. However, the mechanical damage to non-living tissues has not yet been characterized.

Loss of cell viability with microneedle injection must also be considered. Initial experiments testing the shear stress experienced by cells (human fibroblasts) during infusion by microneedles at rates up to 100uL/second showed no loss of viability (Wang, 2006). The study advanced to test the injectibility of 2.8um polymeric microspheres and human intestinal epithelial cells. Results showed a direct correlation between injection pressure and penetration depth; with higher injection pressures, both the cells and microspheres were able to fill the needle track and then exit the needle tip and penetrate the skin (Wang, 2006).

### 1.3 Bulk Cell Seeding Techniques

The aim of tissue engineering is to create structural and functional equivalents for the replacement of diseased anatomy. In order to create a scaffold equivalent to native tissue, the scaffold must be fully repopulated with cells prior to implantation. Bulk seeding aims to obtain an even density of cells throughout the entire thickness of the scaffold.
1.3.1 Bulk vs. Surface Seeding

Cells readily adhere to the exterior surfaces of tissues, but hydrostatic forces prevent passive diffusion of cells into the interior of scaffolds. In order for a cell to move into a scaffold there must be a pathway leading to the interior, sufficient room for the cell to move into, and an affinity or a driving force that allows the cell to migrate to that space.

![Figure 9A: Use of microneedles to obtain bulk seeding](image1)

![Figure 9B: Static seeding using a pipette](image2)

Figures 9A and 9B above compare methods of bulk and static seeding. To conduct bulk seeding using a microneedle, the needle must first pierce the porous scaffold to create a pathway down into the interior of the matrix. The cell suspension can then be injected down into the channel, displacing the water in the hydrated scaffold, and allowing space for the cells to reside. Next, the needle is removed and the cells bind to the peripheral matrix in the channel. Contrarily, in the surface seeding process, the cells are directly pipetted onto the surface of the scaffold. In this method, there is no creation of a pathway or a driving force that promotes cell growth into the interior of the construct. Bulk seeding is much more difficult to accomplish than surface seeding, but is required in order to increase the functionality of the scaffold in vivo.
1.3.2 Requirements of seeding method

In order for a method of cell seeding to be successful, several parameters must be met. The method must not alter the device in any way that would compromise the biocompatibility of the vascular replacement. No chemicals, nanoparticles, or electrostatic charges should alter the function or integration of the graft.

![Diagram of cell seeding criteria]

Figure 10: Criteria for cell seeding

All criteria in Figure 10 must be met in order for the method of cell seeding to be entirely successful.

1.3.3 Current Methods

Numerous methods aimed to drive cells into the middle layers of tissue scaffolds and synthetic grafts have been tested with varying success, yet no method has been
accepted as the gold standard for the cellularization of scaffolds. None of the eight seeding methods listed below have had total, successful results for the bulk seeding of arteries or cusps. If the method does yield a high cellular density, then there exists other negative factors, such as seeding time or complexity, that make it an unsuitable option for seeding. An alternative to these eight methods must be investigated.

**1.3.3.1 Static seeding**

Static seeding has remained the traditional method of seeding for many years due to its simplicity. Static seeding is accomplished by directly pipetting a cellular suspension onto the surface or into the lumen of a graft, and then placing the graft in solution with the cell suspension to allow the cells to passively diffuse onto the tissue. In most cases static seeding results in surface seeding, but no diffusion of cells into the interior of scaffolds. In order to get cells into the interior of tissues, several different approaches have been tested.

**1.3.3.2 Rotational seeding**

In rotational seeding, a graft is rotated in cell/media suspension. Most rotational seeding is conducted with a spinner flask. Spinner flasks are used to quickly and efficiently expand cell lines. Spinner flasks create a turbulent flow within the flask using an impeller blade and magnetic bar. In rotational seeding, the scaffold is placed in the spinner flask with the cell suspension to increase the cell-scaffold interactions.
Rotational seeding provides a higher seeding efficiency compared to static seeding (Kim, 1998). The rotational method of seeding also yields an average of 27% more collagen production when compared to static seeding (Kim, 1998).

Although cell density increases with rotational seeding, the cells remain on the periphery of the scaffolds and demonstrate no significant migration into the intimal layers. Rotational seeding is not an optimal method of cell seeding because a high initial concentration of cells is necessary in order for any significant cell adhesion to occur to the scaffold. (Godbey, 2003)

### 1.3.3.3 Centrifugal seeding

Centrifugal seeding utilizes the same concepts as rotational/dynamic seeding, but incorporates higher centrifugal speeds, reaching up to 6000rpm (Godbey, 2004). The device used for centrifugal seeding is essentially a rotor from a centrifuge secured tightly into a large flask with raised pins that allow rotation in the XY plane. The pins attach to spin tubes, in which the scaffold and cell suspension are deposited.
In an experiment by Godbey et al., 200,000 cells were initially seeded on three identical, polymeric scaffolds and then subjected to static, rotational or centrifugal seeding. After 24 hours, 30,000, 35,000, and 66,000 cells were present on the scaffolds for static, rotational and centrifugal seeding (2500rpm) respectively (Godbey, 2004). Centrifugal seeding had almost two times the cellular retention as rotational seeding.

Centrifugal seeding also facilitated a more homogenous distribution of cells throughout the thickness of the scaffold; however, this homogeneity was highly dependent on the size of cells and the corresponding pore size of the scaffold. If the cell size and pore size were not compatible, then the cellular retention decreased drastically (Godbey 2004). Also, when rotation speeds were increased to promote faster, more forceful cell infiltration, cell lysis occurred.

These constraints prevent centrifugal seeding to be used as a general method of bulk seeding. Centrifugal seeding is only effective when centrifugal speed, scaffold size, cell type, and centrifugation time are optimized for each, individual experiment.

**1.3.3.4 Vacuum seeding**

Apart from rotational seeding, vacuum seeding is the other commonly used method of dynamic seeding. Vacuum seeding uses a pressure differential created by a pump to force a cell suspension through the pores of a graft.
Figure 12: Schematic of vacuum seeding. The cell suspension is pipetted onto the scaffolds and a vacuum pressure is used to pull the suspension through the scaffolds. The vacuum force can be created by a syringe or by a pump. (Li, 2001)

Vacuum seeding has proven to be a quick and efficient method of cell seeding (van Wachem, 1990). The vacuum force traps the cells in the scaffold and increases cellular retention.

Vacuum seeding, when used concurrently with a perfusion system, promotes higher cellular retention and generates an even distribution of cells throughout the length and thickness of the scaffold (Soletti, L). Disadvantages to this method are the complexity of the vacuum/perfusion bioreactor, size limitations of the graft, and the necessity for different seeding procedures with different graft/cell combinations.

1.3.3.5 Electrospun seeding

Electrospinning allows the creation of a scaffold that resembles the composition of the ECM at the sub-micron and nanometer scale. The scaffolds can be designed to influence cell growth and morphology. Studies have shown that cells cultured on aligned scaffolds deposit more ECM than those cultured on disorganized scaffolds (Lee, 2005). This suggests that a scaffold that mimics the ECM will promote greater ECM deposition.
In this method, cells are electrosprayed concurrently with the scaffold polymer to create an electrospun scaffold that has cells inherently located within the scaffold pores. This is accomplished through a set-up using a pump, magnetic fields and voltage fields (Figure 13). The pump simultaneously releases equal amounts of cell solution and polymer solution onto a charged, rotating mandrel, onto which the cells and polymer then form a three-dimensional mesh (Stankus, 2006).

Figure 13: Cellular microintegration. (A) Side by side capillary configuration to electrospray cells onto a flat target moving in an x-y plane. (B) Perpendicular capillary configuration to electrospray cells onto a rotating mandrel moving on a linear stage. (C) The result of the perpendicular capillary configuration. (Stankus, 2006)
Electrospun seeding is both difficult and time consuming. After 45 minutes of electrospaying, the scaffold only achieves a thickness of 100\,\mu m (Stankus, 2010).

The electrospun scaffold cannot survive for extended periods without significant loss of cell viability. Initially after electrospraying, the cells are evenly distributed and healthy, but after 4 days in static or perfusion culture, the cells began to lose viability because the nutrients cannot reach the interior of the matrix (Stankus, 2006). There is also evidence to support that when one cell becomes necrotic, the neighboring cells follow similar apoptotic or necrotic pathways leading to mass cell death in the interior of the scaffold. Also, the effect of the high voltage on cell viability has not been analyzed.

The mechanical properties of the scaffold also suffer with the co-integration of cells into the matrix. The cells disrupt the polymer network and replace the elastic polymer volume with cellular volume, causing a drop in tensile strength and in breaking strain (Stankus, 2006).

1.3.3.6 **Magnetic seeding**

The magnetic seeding method uses ferrous particles to magnetize the cells. The magnetized cells are then drawn into the scaffold by a magnetic force created by the placement of a magnet adjacent to the scaffold. This method of seeding can be accomplished two different ways. The cells can be magnetized by inserting magnetic nanoparticles into the cells, or magnetic particles can be bound to the surface of the cells.

To bind magnetic particles to the exterior of cells, Dynabeads (DynaNiotec, Oslo, Norway) have been used. Dynabeads are superparamagnetic polymer particles that bind to specific cells or proteins. A magnet is inserted into the interior of the vascular graft,
and pulls the beads into the pores of the graft to accomplish seeding efficiencies up to 99% (Perea, 2006). The process of cell seeding can be completed in less than one hour.

To create a magnetic charge within the cell, a different approach is taken. Cells are labeled magnetically using magnetite cationic liposomes (MCLs) and then pulled into the pores of the scaffold using a direct magnetic force.

![Figure 14: Schematic of magnetic seeding.](image)

**Figure 14:** Schematic of magnetic seeding. Cells were labeled with MCL’s then added to culture medium. A magnet was inserted into the center of the scaffold, and then the scaffold was rolled in solution to seed cells evenly. (Shimizu, 2007)

The MCLs are made of a magnetite lipid mixture that results in particle sizes of 150nm. When cells are placed in solution with MCLs, the cells readily absorb the MCLs. Similarly to the Dynabead study; a 99% seeding efficiency is achieved (Shimizu, 2007).

Although promising, magnetic seeding has lost popularity due to the toxic side effects found when excessive amounts of nanoparticles are absorbed by the cell (Ito, 2004). There have not been clear results describing how the magnetite nanoparticles inside of the cell exert their toxic side effects, but cellular assays demonstrate a loss of viability.
Toxicity, in addition to a lack of in vivo studies and extended culture periods of magnetically seeded scaffolds in an in vitro, long-term study, has prevented the use of magnetic cell seeding (Shimizu, 2007).

1.3.3.7 Perfusion/Bioreactor seeding

Studies have shown that mechanical stresses have a beneficial effect on the properties of tissue engineered vascular constructs (Rouwkema, 2010). Bioreactors create a physiological environment that allows for the conditioning of scaffolds to provide a better environment for the survival and growth of the cells in the scaffold (Barron, 2003).

![Bioreactor configuration for cell seeding](image)

**Figure 15: Bioreactor configuration for cell seeding.** Arrows indicate the flow of culture medium through the scaffolds (Kitagawa, 2005)

Bioreactors increase cellular retention by maximizing fluid and mass transfer into the scaffold under physiological pressures. At an experimentally determined optimal perfusion rate, retention of seeded cells over a 5-day period increases by about 7 fold in a bioreactor setting (Kitagawa, 2005).
Although effective when optimized, seeding outcomes deteriorate when the concentration of cell suspension and flow rate are not experimentally determined for each combination of scaffold and cell type. The need to optimize the process in an already complex bioreactor system causes this method of cell seeding to be extremely difficult. This method is also time consuming, as it requires about a week for cells to adhere and migrate into all layers of the tissue under continual flow conditions.

### 1.3.3.8 Scaffold Coating

In order to get cells to readily bind to scaffolds, efforts have been made to coat scaffolds with different biological glues such as fibrin and fibronectin, or with ECM proteins such as collagen or laminin (Salacinski, 2001; Pawlowski, 2003). The goal of scaffold coating is to bind the cells to the matrix or to trap the cells in the matrix. Scaffold coating limits seeding to the periphery of the grafts and does not encourage cell migration into the graft. Other concerns with scaffold coating are that cells will form conglomerates where the proteins are bound, and not coat the free surfaces of the graft. This may provide a site for thrombosis and clot formation.

### 1.3.4 Partial Conclusion

Although the various methods aforementioned demonstrate an increase in cell density in the interior of scaffolds, each method is flawed, often requiring long incubation times, complex set-ups, or damages to cell viability and the mechanical properties of the scaffold.
Figure 16: Summary of existing seeding methods including advantages and disadvantages

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static Seeding</td>
<td>Cells pipetted onto scaffold</td>
<td>Cheap, simple</td>
<td>Surface seeding</td>
</tr>
<tr>
<td>Rotational Seeding</td>
<td>Spinner flasks mix the scaffold and cells together</td>
<td>Increased cell-scaffold contact</td>
<td>Slow, surface seeding</td>
</tr>
<tr>
<td>Centrifugal Seeding</td>
<td>A device equipped with a centrifuge spins cells from a center tube out to the surrounding tissue at high speeds</td>
<td>Increased cell penetration</td>
<td>Shear damage to cells, only for small scaffolds</td>
</tr>
<tr>
<td>Vacuum Seeding</td>
<td>A vacuum force is used to pull cells down through the pores of the scaffold</td>
<td>Simple</td>
<td>Only works for simple, flat anatomy</td>
</tr>
<tr>
<td>Electroseeding</td>
<td>While electrospinning the polymer scaffold, an adjacent reservoir sprays a cell solution into the scaffold</td>
<td>Cells innately embedded in scaffold</td>
<td>Lack of research Long term loss of cell viability</td>
</tr>
<tr>
<td>Magnetic Cell Seeding</td>
<td>Incorporates magnetic microparticles into cells and uses a magnet to promote cell infiltration</td>
<td>High seeding efficiency</td>
<td>Toxicity No long term studies</td>
</tr>
<tr>
<td>Perfusion Bioreactor based seeding</td>
<td>Mimics physiological conditions to maximize cell morphology and ECM remodeling</td>
<td>Conditions cells</td>
<td>Complex, long time, Need for optimization</td>
</tr>
<tr>
<td>Scaffold Coating</td>
<td>Increases cells affinity to bind to scaffolds through binding of native proteins to the scaffold</td>
<td>Increased binding to cell surface</td>
<td>Limited penetration Aggregates of cells</td>
</tr>
</tbody>
</table>

We believe that an injection method of cell seeding will allow us to physically repopulate the scaffold to a natural cell density while also limiting the adverse effects associated with existing cell seeding methods.
CHAPTER 2: PROJECT APPROACH

2.1 Rationale

In order for a tissue-engineered substitute to be successful, it must not only mimic the native tissue but must also fully integrate into a living system. When a scaffold is implanted without cells present both externally and internally, thrombosis and clotting occur along the graft. No seeding method exists to date that can fully cellularize a tissue in a short amount of time without causing mechanical or cellular damage to the construct. Our group believes that an injection method of seeding is the best approach for the reseeding of decellularized tissues.

The goal of this master’s project has been to design and test different configurations of microneedle arrays to determine if these arrays are a suitable option for the reseeding of scaffolds. This is the first study to determine if microneedles have the potential to seed scaffolds via microinjection.

2.2 Specific Aims

This project has three major goals: 1) Design or purchase several variations of microneedle arrays that vary in needle count, height, and diameter and 2) Test the ability of the microneedles to confluent seed the entire thickness of a scaffold 3) Determine the effect that repeated insertion of microneedles has on the mechanical integrity of the graft.
2.2.1 Aim I: Design/purchase microneedle arrays

The microneedle array had three top design requirements: That (1) the needle bores be large enough to allow the passage of cell suspension without damaging the cells (2) the needle array sharply pierce the tissue repeatedly without damaging the needles, and (3) the needle lengths not exceed the thickness of the tissue. All materials were to be non-toxic and sterilizable.

2.2.2 Aim II: Experimentally validate the injection method

The ability of the microneedles to deposit cells into the medial layers of the scaffold was tested on two different cardiovascular tissues: aortic cusps and carotid arteries.

Three decellularized aortic cusps and three decellularized carotid arteries were seeded using each of the three different microneedle arrays. A fourth decellularized cusp and a fourth decellularized artery was seeded using a hypodermic needle as a control. The tissues were incubated in DMEM for 8 hours to allow cells to adhere and to promote surface seeding.

Cellular assays including DAPI, Massons Trichrome, and H&E staining were used to evaluate cell density and migration.

2.2.3 Aim III: Quantify mechanical damage

Repeated insertion of the needles into the tissue may cause significant damage to the mechanical integrity of the scaffold. To determine if the damage was significant, a uniaxial tensile test was conducted to compare mechanical differences within fresh
tissues, decellularized tissues, and decellularized punctured tissues. The carotid arteries were further tested with a burst pressure test, suture retention test and compliance test to determine if the scaffolds were still capable of withstanding physiological internal pressures.
CHAPTER 3: MATERIALS AND METHODS

3.1 Microneedle Design

A set of design criteria was composed to determine the necessary characteristics and design specifications of the microneedle array.

![Diagram showing design criteria for microneedle arrays]

Figure 17: Design criteria for microneedle arrays

Three different microneedle arrays were tested in this study: MicronJet (Nanopass, Israel), Hollow microneedle Hub (Micropoint Technologies, Singapore), AdminPen (AdminMed, California).
Figures 18 A, B, C: Gross images of the selected microneedle arrays (a) Micropoint array: 3x3, 1mm height, 300µmx300µm base hollow microneedle hub; made of stainless steel (b) AdminPen: one hundred eighty seven 500 µm-tall microneedles on 1cm² circular array; made of SS316 stainless steel (c) MicronJet: three 600µm-tall microneedles; made of silicon

Before these devices were obtained, we attempted to 3D print a microneedle array. A syringe-attachable microneedle array was designed in Solidworks and sent to the Clemson University machine shop for fabrication.

The Objet350 Connex with a resolution of 16um was used to print the device out of VeroWhite. Many different printing configurations and different materials were attempted, but the needles were unable to be printed with hollow bores or sufficiently sharp tips.
3.2 Experimental Methods

The following protocols were used in the experimentation, testing, and analysis of microneedle injection of cells into arteries and leaflets.

3.2.1 Carotid Artery Scaffold Preparation

Fresh carotid arteries 5–10 cm long, tapering from 6 to 3mm (Animal Technologies, Tyler, TX) from young porcine (6-8 weeks) were decellularized using a novel bioprocessing system designed by colleague, Dr. George Fercana.

Figure 20: Double reservoir bioprocessing system with 12 mounted porcine carotid arteries. The arteries are pressurized at 80mmHg with continuously circulating decellularization solutions.
3.2.1.1 Cleaning and Preparation

Once received, the carotid arteries were cleaned over ice to remove excess fat. Luer lock fasteners were zip tied to each end of the arteries for future mounting into the bioprocessing system. To test for leaks, one luer lock was closed off with a luer cap while the other end of the artery was tightened onto a syringe filled with saline. The arteries were pressurized and monitored for any leaks. If a leak was observed, the hole was sutured with standard suturing procedures using a 3-0 Ethibond Excel braided polyester suture.

Before initiating the decellularization processes, samples were treated in 30 mM EDTA, 0.02% Sodium Azide (NaN₃) and pH to ~7, then placed on an orbital shaker at 3.5 rpm at 4°C to remove gross bioburden and kill bacteria.

3.2.1.2 Tissue Decellularization and Sterilization

The fresh arteries were subjected to a series of detergents and chemical solutions to remove cells and cellular debris. Ethylenediaminetetraacetic acid (EDTA) was used first in the decellularization process. EDTA is a chelating agent that disrupts cell adhesion to ECM. EDTA works most effectively with sodium dodecyl sulfate (SDS), which is used to remove nuclei from tissues through a mechanism that is disruptive to ECM.

Following SDS treatment, sodium hydroxide (NaOH) was used to eliminate growth factors from the matrix. NaOH is used in short time increments because it disrupts collagen crosslinking. Ethanol was next used to lyse cells by dehydration. Post ethanol treatment, RNase and DNase are used to catalyze the hydrolysis of
ribonucleotide and deoxyribonucleotide chains. Peracetic acid was used last as disinfection agent that doubles as a decell agent by removing residual nucleic acids with minimal effect on the ECM composition and structure.

Materials

- 30 mM EDTA, 0.02% Sodium Azide (NaN₃) (pH to 7.0)
- 1% SDS solution in water
- 0.1 M NaOH solution
- 70% ethanol (EtOH)
- ddH₂O for rinsing
- 1x PBS
- 0.1% v/v peracetic acid solution in 1x PBS at pH 7.0
- Sterile water

Methods

1. Place samples in 30 mM EDTA, 0.02% Sodium Azide (NaN₃) on an orbital shaker at 3.5 rpm in the cold room overnight
2. Mount the arteries in the perfusion bioreactor (Fercana)
3. Fill the bioreactor with 30 mM EDTA, 0.02% Sodium Azide (NaN₃) solution and leave overnight
4. Begin ddH₂O protocol

**ddH₂O protocol**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>16L ddH₂O</td>
<td>1. Flush 0.5L ddH₂O through each reservoir, then clamp off the system and add another 0.5L to allow the partial mixing before drainage. Drain the water</td>
</tr>
<tr>
<td></td>
<td>2. Refill each reservoir with 1L of ddH₂O and pressurize the system to allow the water to rinse the entire system. Drain the system</td>
</tr>
<tr>
<td></td>
<td>3. Repeat steps 1 and 2 three more times</td>
</tr>
</tbody>
</table>

5. After the ddH₂O protocol, start draining the water.
6. Introduce the 1% SDS solution into the reservoir in 500 mL increments.
   a. Incubate for 12 days.
   b. Change 1% SDS solution after 6 days.
7. After 12 days, conduct ddH₂O Rinse Protocol
8. Introduce ddH₂O as an overnight rinse step with pressure.
9. Conduct three – 1 L infusions of 70% ethanol (EtOH):
   a. Introduce 70% EtOH in 500 mL increments; after 1 L introduced, pressurize system and close waste circuit to allow 70% EtOH to circulate with pressure for 1 hour.
   b. Repeat ‘a’ 2 more times for a total of 3 hours of 70% EtOH circulation with pressure.
10. Repeat ddH₂O Rinse Protocol. Using the last 500 mL of
11. Rinse with ddH₂O for 30 min with pressure.
12. While draining water out of system and into waste carboy, introduce the 0.1M NaOH solution into the reservoir in 500 mL increments.
   a. After 1400 mL of 0.1M NaOH introduced into system, incubate for 2 hours with pressure
13. After 2 hours, conduct ddH$_2$O Rinse Protocol
14. Conduct one – 1400 mL infusion of 1x PBS:
   a. Introduce 1x PBS in 500 mL increments; after 1400 mL is introduced, pressurize system and close waste circuit to allow 1x PBS to circulate with pressure for overnight.
15. Treat scaffolds with DNAsse/RNAsse solution for 96 hours at room temperature.
   a. 720 mUnits/mL DNAsse, 720 mUnits/mL RNAsse, 5 mM MgCl, in 1x PBS – adjust pH to 7.5
   b. Add antibiotic/antimycotic 1% to prevent contamination during this step.
16. After DNAsse/RNAsse treatment, conduct one – 1400 mL infusion of 1x PBS:
   a. Introduce 1x PBS in 500 mL increments; after 1400 mL is introduced, pressurize system and close waste circuit to allow 1x PBS to circulate with pressure for 1 hour.

MOVE SYSTEM INTO A STERILE HOOD

17. Conduct ddH$_2$O water rinsing protocol – should not be overnight; need PBS to neutralize acid on same day.
18. Conduct two – 1400 mL infusions of 1x PBS (sterile), the last 500 mL will circulate with pressure for 20 min.
19. Place in peracetic acid, the last 500 mL will circulate for 2 hours with pressure, room temperature
   a. Must shake reservoir and dampener (capped) vigorously for 1 minute each, once an hour to ensure sterility of each bottle.

3.2.1.3 Tissue Fixation

Following decellularization and sterilization, scaffolds were stabilized with sterile-filtered 0.15 % pentagalloyl glucose (PGG, N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium) in 50 mM dibasic sodium phosphate buffer with 20% isopropanol in saline, at pH 5.5 and room temperature, without perfusion or pressure, for 24 hours.

Materials

- 0.15% w/v PGG solution in 50 mM HEPES pH 5.5 with 20% isopropanol. Sterile filter before use.
- 70% ethanol (EtOH) (One – 1400 mL infusion per bioreactor)

Methods

1. Fill the whole system with sterile PGG. Treat for 24 hours at room temperature, no perfusion, and no added pressure.
2. Next day, administer two – 1400 mL infusions of 1x sterile PBS; the last 500 mL administered will circulate, with pressure, for 10 minutes.
3. Administer one – 1400 mL infusion of 70% ethanol; the last 500 mL to circulate with pressure for 10 min.
4. Conduct ddH₂O rinsing protocol with sterile water
5. Administer one – 1400 mL infusion of 1x sterile PBS, with the last 500 mL circulating with pressure, for 24 hours.
6. Repeat this step daily for a total of 6 days.
7. In sterile hood: remove arteries, cut off barbed adaptors.
8. Store arteries in sterile 1x PBS + 1% antibiotics/antimycotics at 4°C

3.2.2 Aortic Cusp Scaffold Preparation

The aortic cusps were decellularized with a thirteen-day immersion decellularization process.

3.2.2.1 Cleaning and Preparation

Aortic valves were removed from fresh porcine hearts, and collected from a local USDA approved abattoir, Snow Creek Meat Processing in Seneca, SC. Aortic roots were dissected up to sino-tubular junction, with 5-10 mm long coronaries, and down to 5-10 mm beyond the base of the cusps. Valves were collectively stored in a 500mL bottle of ddH₂O over ice during transportation to the laboratory. Fat and extraneous tissue were cut away from the aortic root. The root was rinsed 3 times in ddH₂O, and then incubated in ddH₂O overnight at 4°C.

3.2.2.1 Tissue Decellularization and Sterilization

The bulk of cellular removal was accomplished with sodium hydroxide and an SDS/TRIS solution. After this, the detergents were rinsed from the arteries with PBS and sterile water. RNAse and DNase were then used to remove any remnants of cellular
debris. These solutions were then rinsed away with PBS to prepare for tissue sterilization. The sterilization was accomplished with peracetic acid.

Materials

- ddH2O
- 0.05 M NaOH [Fisher BP359-500]
- Decell solution (need 1000 mL)
  - 50 mM TRIS [EMD TX1529-5]
  - 0.25% SDS [JT Baker 4095-02]
  - 0.5% DOC [Fisher BP349-100]
  - 0.5% Triton X100 [Alfa Aesar A16046]
  - 0.2% EDTA [Sigma Aldrich E6511]
  - pH to 7.4±0.05
  - fill to 1000 mL with ddH2O
- 0.02% Sodium azide [Fisher S227I-100]
- 70% EtOH
- 1xDPBS [from 10x – Mediatech 20-031-CV] (need 6-8 L) = 1x concentration, no calcium or magnesium
- 0.02% Sodium azide in 1xDPBS
- DNase/RNase
  - 0.02% NaN3
  - 5mM MgCl2 [Fisher M33-500]
  - 360 mU/mL DNase (Worthington cat#: 2139 lot#: S7D9604A, 3480u/mgDW)
  - 360 mU/mL RNase (Fisher cat#: BP2539250 lot#: 131006, 97.1 KUNITZ/mg)
  - fill to 2 L with 1xDPBS
- Sterile 1xDPBS = 1x concentration, no calcium or magnesium
- Sterile 0.1% Peracetic acid [Sigma Aldrich 269336]
- Sterile cotton balls

Methods

1. Rinse with ddH2O (3x)
2. Incubate in 0.05M NaOH 2 hour at room temperature on a shaker
   a. Start timer for 2 hours
3. Rinse with ddH2O (3x)
4. Incubate in ddH2O 15 minutes at room temperature on a shaker (3x)
5. Incubate in decellularization solution 48±3 hours at room temperature on a shaker
6. Rinse with ddH2O (5x)
7. Incubate in ddH2O 5 minutes at room temperature on a shaker (5x)
8. Incubate in 0.02% Sodium azide 20±2 hours at room temperature on a shaker
9. Rinse with ddH2O (3x)
10. Incubate in 70% EtOH 2 hours at room temperature on a shaker
11. Replace with fresh 70% EtOH and incubate 2 hours at room temperature on a shaker
12. Rinse with ddH2O (2x)
13. Incubate in ddH2O 15 minutes at room temperature on a shaker (3x)
14. Incubate in 0.02% Sodium azide 20±2 hours at room temperature on a shaker
15. Incubate in 1xDPBS 5 minutes at room temperature on a shaker (3x)
16. Incubate in 0.02% Sodium azide in 1xDPBS 24±2 hours at room temperature on a shaker
17. Incubate in 1xDPBS 5 minutes at room temperature on a shaker (3x)
18. Incubate in DNase/RNase solution 24±2 hours at 37°C (oven, incubator, or warm room) on a shaker  
   a. Note: ensure that the DNase/RNase solution is pre-warmed  
19. Replace with fresh DNase/RNase solution and incubate 24±2 hours at 37°C on a shaker  
20. Rinse with 1xDPBS (2x)  
21. Incubate in 1xDPBS 15 minutes at room temperature on a shaker (3x)  
22. Incubate in 0.02% Sodium azide in 1xDPBS 24±2 hours at room temperature on a shaker  
23. Transfer valves into new, sterile jars  
24. Fill jars with sterile 0.1% Peracetic acid and incubate for 2 hours at room temperature on a shaker  
25. Aspirate jars until empty and rinse with sterile 1xDPBS  
26. Aspirate jars until empty and incubate in sterile 1xDPBS for 1 hour at room temperature on a shaker  
27. Aspirate jars until empty and incubate in sterile 1xDPBS for 2 hours at room temperature on a shaker  

3.2.2.2 Tissue Fixation

Following decellularization and sterilization, scaffolds were stabilized with sterile-filtered 0.15 % pentagalloyl glucose (PGG, N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium) in 50 mM dibasic sodium phosphate buffer with 20% isopropanol in saline, at pH 5.5 and room temperature for 21 hours. The scaffolds were then rinsed and stored in sterile PBS.

Materials

- Sterile cotton balls (need about 15-20 pieces for 5 valves) = autoclaved  
- 0.15% PGG solution (need 500 mL)  
  o For PGG stock: 0.15% PGG (0.75 g for 500 mL) dissolved in 100 mL isopropanol;  
  o For phosphate buffer: 50 mM Na₂HPO₄ (2.84 g for 500 mL), 0.9% NaCl (3.6 g for 500 mL), in 350 mL ddH₂O, pH to 5.5±0.05, fill to 400 mL with ddH₂O;  
  o To make 0.15% PGG solution: slowly add 100 mL PGG stock to the 400 mL phosphate buffer under constant stirring, run through 0.22 µM sterile filter into a sterile bottle  
  o Protect from light by wrapping bottle in aluminum foil

Methods

1. Aspirate jars until empty and pack up cusps with PGG-soaked sterile cotton balls  
2. Incubate in 0.15% PGG for 21±1 hours on shaker wrapped in aluminum foil  
3. Aspirate jars until empty and remove cotton balls with sterile tools and discard  
4. Rinse with sterile 1xDPBS  
5. Aspirate jars until empty and incubate in sterile 1xDPBS for 1 hour on shaker  
6. Aspirate jars until empty and incubate in sterile 1xDPBS for 19±1 hours on shaker  
7. Aspirate jars until empty
8. Transfer remaining valves to sterile 50 mL conical tubes (maximum of 4 per tube)
9. Store valves in sterile 1xDPBS at 4°C

3.2.3 Cell Culture

Human adipose derived stem cells (hADSCs) were subcultured in DMEM supplemented with 10%FBS and 1% antibiotic/antimycotic. Cells were incubated in a humidified atmosphere at 95% air and 5% CO₂ at 37°C. Monolayer cultured hADSCs at the passage numbers of 6-11 were harvested with trypsin and used for experiments.

3.2.4 Cell Seeding

The carotid arteries were cut down to 2cm cylinders. hADSCs were suspended at 4x10⁶ cells/mL in culture media and 400 µL were injected through the adventitia into the artery with a syringe and repeating dispenser (Hamilton Company, Reno, Nevada) using 10 µL of cell suspension per injection, and applying an even pattern of injections across the artery exterior. Each time the needles were inserted, the dispenser was pressed 2 or 3 times to ensure the cells entered the tissue. The artery was cultured in static conditions for 8 hours in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and 1% antibiotic solution (Corning-Cellgro).

For the aortic cusps, hADSCs were suspended at 4x10⁶ cells/mL in culture media and 500 µL were injected into each cusp with a syringe and repeating dispenser (Hamilton Company, Reno, Nevada) using 10 µL of cell suspension per injection, and applying an even pattern of injections across both sides of the cusp. Each time the needles were inserted, the dispenser was pressed 2 or 3 times to ensure the cells entered the tissue. The cusp was cultured in static conditions for 8 hours in Dulbecco’s modified
Eagle’s medium.

### 3.2.5 Hematoxylin and Eosin

Hematoxylin & Eosin (H&E) staining is a customary staining method in histology. It uses the basic dye, hematoxylin, to color basophilic structures a blue-purple hue. The acidic dye, eosin, colors eosinophilic structures, such as cytoplasm and some extracellular matrix products, bright pink (Ross, Kaye et al., 2003).

Following fixation in 10% formalin, cusps and arteries were cut in half, embedded in paraffin wax, sectioned, and stained according to the following routine H&E staining method.

1. Xylene ------------ 10 dips
2. Xylene ------------- 5 min
3. 100% EtOH ----------- 10 dips
4. 100% EtOH ------------ 1 min
5. 95% EtOH ------------ 10 dips
6. 95% EtOH ----------- 1 min
7. Running Water -------Till Clear
8. Distilled Water ------ 1 min
9. Hematoxylin --------- 5 min
10. Running Water -------Till Clear
11. Clarifier 10-15 dips
12. Running Water -------Till Clear
13. Bluing 1 min
14. Running Water ------ 30 sec
15. 95% EtOH ----------- 10 dips
16. Eosin 45 sec
17. 95% EtOH ----------- 10 dips
18. 95% EtOH 10 dips
19. 100% EtOH 10 dips
20. 100% EtOH 10 dips
21. 100% EtOH 10 dips
22. Xylene 10 dips
23. Xylene 5 min
3.2.6 DAPI

DAPI (4’-6-Diamidino-2-phenylindole) is a fluorescent stain that will pass through an intact cell membrane and bind strongly to double stranded DNA. It is the standard in fluorescence microscopy to stain both live and fixed cells with an emission of bright blue fluorescence (Invitrogen, 2006).

After performing routine H&E staining, DAPI staining helped validate the presence of nucleic acids that were difficult to distinguish. Each fixed and paraffin-embedded section was brought back to water according to the following steps and stained directly on the slide.

1. Xylene -------------- 10 dips
2. Xylene -------------- 5 min
3. 100% EtOH ---------- 10 dips
4. 100% EtOH ---------- 1 min
5. 95% EtOH --------- 10 dips
6. 95% EtOH --------- 1 min
7. Running Water -------Till Clear

3.2.7 Massons Trichrome

Massons trichrome is a stain for collagen fibers. Collagen stains dark blue, nuclei stain black, and muscle and cytoplasm stain pink. After performing other routine staining, Massons Trichrome staining helped distinguish the damage to the collagen in the scaffold due to the forced insertion of the microneedles. Each fixed and paraffin-embedded section was rehydrated and stained according to the following routine Massons Trichrome staining procedure.

1. Deparaffinize and rehydrate through 100% alcohol, 95% alcohol 70% alcohol.
2. Wash in distilled water.
3. Re-fix in Bouin's solution for 1 hour at 56 C to improve staining quality
4. Rinse running tap water for 5-10 minutes to remove the yellow color

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5. Stain in Weigert's iron hematoxylin working solution for 10 minutes
6. Rinse in running warm tap water for 10 minutes
7. Wash in distilled water
8. Stain in Biebrich scarlet-acid fuchsin solution for 10-15 minutes. Solution can be saved for future use
9. Wash in distilled water.
10. Differentiate in phosphomolybdic-phosphotungstic acid solution for 10-15 minutes or until collagen is not red.
11. Transfer sections directly (without rinse) to aniline blue solution and stain for 5-10 minutes.
12. Rinse briefly in distilled water and differentiate in 1% acetic acid solution for 2-5 minutes.
13. Wash in distilled water.
14. Dehydrate very quickly through 95% ethyl alcohol, absolute ethyl alcohol (these step will wipe off Biebrich scarlet-acid fuchsin staining) and clear in xylene.
15. Mount with resinous mounting medium.

3.2.8 Uniaxial Tensile Testing

Uniaxial tensile testing determines the yield stress and the elastic modulus of the specimen. This test is standardized in accordance with the American National Standards Institute/Association for the Advancement of Medical Instrumentation (ANSI/AAMI) Section 7198:8.3.2. To test the arteries, the tubular scaffold was cut down to a length of 2 cm (n = 3 per group) and both ends were clamped to the jaws of a 100N MTS test frame (MTS Systems Corp., Eden Prarie, MN). The sample was then stretched at a uniform rate of 10 mm/min until the yield and/or break point was reached. Throughout the test procedure the artery was continually wetted with PBS. The load at yield or break was recorded.

To test the cusps, a rectangle was cut from the cusp. The length of the cusp was maximized in the circumferential direction. The thickness and width of the cusp was measured and both ends of the rectangular section were clamped into the jaws of the 100N MTS test frame. The sample was then stretched at a uniform rate of 10 mm/min
until the yield and/or break point was reached. Throughout the test procedure the cusp was continually wetted with PBS.

### 3.2.9 Suture Retention Testing- Carotid Arteries

Suture retention strength calculates the anastomotic strength of an arterial graft. This test is standardized in accordance with the American National Standards Institute/Association for the Advancement of Medical Instrumentation (ANSI/AAMI) section 7198:8.8. Suture retention strength was calculated by cutting arterial scaffolds (n = 4 per group) normal to the long axis into 6x20mm segments, clamping one end to an 100N MTS test frame (MTS Systems Corp., Eden Prairie, MN), and placing a single 3-0 braided polyester suture 2mm from the free edge and looped around the test frame. Sections were then preloaded to 0.009N and extended to failure at 5mm/min and final data expressed as grams-force. The force required to pull the suture through the prosthesis or cause the wall of the prosthesis to fail was recorded.

### 3.2.10 Burst Pressure- Carotid Arteries

Burst pressure is important when determining the maximum amount of pressure an artery can withstand without breakage. This test is standardized in accordance with the American National Standards Institute/Association for the Advancement of Medical Instrumentation (ANSI/AAMI) Section 7198:8.3.3.

Using the calipers, the wall thickness of the vascular scaffold on both ends as well as the total length of the vascular scaffold was measured. An appropriately sized luer fitting was placed into vascular scaffold’s lumen, one side at a time, and secured with a 3-
braided polyester suture and then fastened with a zip tie. The artery was cut so that there was 2.0cm between the ends of the proximal and distal fitting. A circuit with a peristaltic pump, pressure transducer, and distilled water fluid reservoir was built. A data acquisition device was attached to the pressure transducer (DAQ) to allow accurate reading of the transducer. The pump was turned on and the internal pressure at which the artery burst was retrieved by DAQ software. For each condition n = 3 specimens were tested.

**3.2.11 Compliance- Carotid Arteries**

Diametrical compliance testing required a syringe pump and pressure transducer in addition to a digital camera. A 50mL luer lock syringe was filled with distilled water and connected to the artery that was connected to the pressure transducer. A metric ruler was secured next beneath the artery to act as a scale for later interpretation of the change in diameter. To determine compliance, arterial scaffold segments were exposed to 80mmHg then 120mmHg. At each pressure a digital image of the artery was taken. Images were then imported into Image J software (National Institutes of Health, Bethesda, MD) to measure external diameters at three positions perpendicular to the scaffold margins for each image. Diametrical compliance was then calculated by inputting mean values into to equations proposed by Hamilton’s group and expressed as percentage distension per 100mmHg (n = 3 per group).
3.3 Experimentation

The results of the microneedle seeding study are shown below. The histological results compare the seeding results of a 30-gauge needle to the Micropoint 9-microneedle hub. Only the Micropoint Technology, 9-needle hub was used in the study due to the failure of the other two devices. The Nanopass, MicronJet array shattered upon contact with the first tissue and the AdminMed, AdminPen was incapable of delivering fluid through the needle bores. These failures will be addressed in further detail in the discussion.

3.3.1 Carotid Artery

3.3.1.1 Tissue Decellularization

To verify the decellularization of the tissue, H&E was used to both detect the presence of nuclei and to visualize any potential damage of the ECM due to the decellularization process. Figure 21D shows the structure and composition of a fresh, porcine carotid artery prior to decellularization and Figure 21A and Figure 21D show the artery after decellularization. No nuclei were seen in the H&E sections and the organization of the extracellular matrix remained in tact.

In addition to histology, ethidium bromide agarose gel electrophoresis was performed to separate fragments of DNA for quantification (Figure 21C).
Figure 21: Verification of decellularization process. (a,d) H&E stain of decellularized porcine carotid artery showing an organized ECM structure and no presence of cells. Collagen=light purple/pink and elastin=dark purple/pink (b) H&E stain of a fresh porcine carotid artery with a high nucleic acid content. Nuclei=dark purple (c) Image of electrophoresis gel. Nucleic acids emit red light. The two left columns are fresh carotid arteries and the seven columns to the right of those are decellularized carotid arteries.

The absence of red light in the right seven lanes of the gel indicates that decellularization is complete. Although there is no direct numerical quantification of the remaining DNA with this method, the gross visualization associated with electrophoresis is accurate enough to determine the overall success of decellularization.
3.3.1.2 **DAPI**

DAPI was used to detect the presence of nuclei. A high number of blue stained nuclei represent a large number of cells seeded within the scaffold (Figure 22A and Figure 22B).

![Figure 22: DAPI staining to compare cellular presence after injection with 30G and 9-microneedle hub. Nucleus=blue. (a,b) Injection with a 30 Gauge needle (c,d) Injection with 9-microneedle hub](image)

With the 30G needle, twenty 10uL boluses were pushed into the media of the artery. In (a) a single cell can be seen in the bottom right and (b) shows a few cells in the middle of the slide but overall there is a low cellular density with the 30G needle. The 9-microneedle hub produced a slightly higher cell density, but there is minimal cellular infiltration overall.
3.3.1.3  

**H&E**

Cells were not populous after seeding with the 9-microneedle hub or with the 30-gauge needle. In Figure 23A, there are some cells lining the lumen of the artery, and also located in the pocket in the tunica media. It is uncertain whether the needle created this pocket. In Figures 23B and 23C there are cells both internally and externally lining the surfaces of the arteries. Surface seeding is not intended with the use of microneedles.

![Figure 23](image.png)

**Figure 23:** H&E staining to compare cellular presence after injection with 30G and 9-microneedle hub. (a) Injection with 30G needle (b,c) Injection with 9-microneedle hub

The break in the adventitia shown in Figure 23C may be indicative of where the microneedle penetrated the artery. Around this site, there appears to be a larger number of cells compared to other locations.
3.3.1.4 **Massons Trichrome**

Surface seeding along the lumen and adventitia of the arteries is noted, but there is no visible cellular infiltration in either of the stains (Figure 24A and Figure 24B). However, the presence of cells may go undetected since they stain the same reddish color as the elastin.

![Figure 24](image)

**Figure 24:** Massons Trichrome staining to detect cellular presence after injection. **Collagen = blue,** nuclei = dark red/ purple, cytoplasm = red/pink (a) Injection with 30G needle (b) Injection with 9-microneedle hub

3.3.1.5 **Uniaxial Testing**

A tukey test in conjunction with an ANOVA to determine means that are significantly different from each other was performed for each mechanical test using SAS Studio (SAS, Cary, NC). Results showed that there is no statistically significant difference in the ultimate tensile stress or in Young’s modulus amongst fresh tissues, decellularized tissues, and decellularized tissues with repeated injections of PBS.
Figure 25: Uniaxial tensile testing to compare fresh, decell, and decell+needle scaffolds. ^ indicates that the value was derived from literature in the field. (a) Ultimate tensile strength (n=2 Decell, n=2 Decell+Needle) ^ from Campbell, 2005 (b) Young’s Modulus (n=2 Decell, n=2 Decell+Needle) ^ from Campbell, 2005 (c) Stress-strain curve used to determine ultimate tensile stress and young’s modulus

It can be concluded that needle piercings through the cusp have no significant effect on the mechanical strength of the arterial scaffold.

3.3.1.6 Suture Retention

Figure 26 shows the suture retention strength of fresh arteries, decellularized arteries, and decellularized arteries with microneedle injections of 1X PBS. The suture retention
strength of the decellularized scaffolds exceeds that of a fresh artery. No statistically significant difference was found amongst the three groups.

**Figure 26: Suture retention strength between fresh, decell, and decell+needle scaffolds.** ^ indicates that the value was derived from literature in the field. (a) Initial loading and preconditioning of the sample into the MTS machine (b) Failure point of the suture retention test (c) Suture retention strength (n=4 Decell, n=4 Decell+Needle) ^ from Conklin, 2002

### 3.3.1.7 Burst Pressure

Figure 27 shows the burst pressures of fresh arteries, decellularized arteries, and decellularized arteries with microneedle injections of 1X PBS. The burst pressures of the decellularized scaffolds exceed that of a fresh artery. No statistically significant difference was found amongst the three groups.
Figure 27: Burst pressures between fresh, decell, and decell+needle scaffolds. ^ indicates that the value was derived from literature in the field. (a) Testing apparatus to determine burst pressure (b) Burst pressure (n=3 Decell, n=2 Decell+Needle) ^ from Conklin, 2002; Cohut, 2001; Roeder, 2001

3.3.1.8 Compliance

Compliance was defined as the inverse of Peterson's elastic modulus according to the following equation: $C = (\Delta D/D_0)/\Delta P$. A tukey test in conjunction with an ANOVA to find means that are significantly different from each other (p<0.05) was performed, and results showed that decell and decell+needle are both significantly different from the fresh artery. Trends in literature agree with this finding. Compliance tends to decreases after tissue decellularization.
3.3.2 Aortic Cusp

3.3.2.1 Tissue Decellularization

Verification of decellularization was completed with histological staining. H&E was used to both detect the presence of nuclei and to visualize any potential damage to the ECM due to the decellularization process. Figure 29A shows the structure and composition of a fresh aortic cusp prior to decellularization and Figure 29B show the artery post decellularization. No nuclei were seen in the decellularized H&E sections, and the organization of the extracellular matrix appeared undamaged.

DAPI stains nuclei bright, fluorescent blue. All of the nuclei present in Figure 29C are removed in Figure 29D by the decellularization process.
Figure 29: Histological stains to verify decellularization (a) H&E stain of fresh porcine aortic cusp (b) H&E stain of decellularized porcine aortic cusp (c) DAPI stain of fresh porcine aortic cusp nuclei=blue (d) DAPI stain of decellularized porcine aortic cusp nuclei=blue

3.3.2.2 H&E

Cells are visible after seeding with the 9-microneedle hub and with the 30-gauge needle. In Figure 30B, there appears to be some mechanical damage to the ECM of the cusp. It is uncertain whether the needle created this damage. In Figures 30C and 30D there is an abundance of cells within the pores of the scaffolds. The cells seem evenly dispersed throughout the scaffold.
Figure 30: H&E staining to compare cellular presence after injection (a,b) Injection with 30G needle (c,d) Injection with 9-microneedle hub

3.3.2.3 DAPI

Figure 31B shows a large cluster of cells distributed through the tissue. The tissue in Figure 31A fluoresces brightly, but there are few defined nuclei.
Figure 31: DAPI staining to compare cellular presence after injection with 30G and 9-microneedle hub. Nucleus=blue. (a) Injection with a 30 Gauge needle (b) Injection with 9-microneedle hub

3.3.2.4 Massons Trichrome

Unlike the staining of the arteries, the cells in the cusps can be easily visualized with Massons Trichrome. Figures 32C and 32D show cellular infiltration of the pores of the cusps. Figure 32A shows some cells within the scaffold as well as along the periphery of the scaffold.
3.3.2.5 **Uniaxial Testing**

Results of statistical testing show no statistically significant difference in the ultimate tensile stress or Young’s modulus amongst fresh tissues, decellularized tissues, and decellularized tissues with repeated injections of PBS. It can be concluded that repeated needle piercings through the cusp have no significant effect on the mechanical strength of the cusp.
Figure 33: Uniaxial tensile testing to compare fresh, decell, and decell+needle scaffolds. ^ indicates that the value was derived from literature in the field. (a) Ultimate tensile strength (n=2 Decell, n=2 Decell+Needle) ^ from Clark, 1971 (b) Young’s Modulus (n=2 Decell, n=2 Decell+Needle) ^ from Clark, 1971 (c) Stress strain curve used to determine ultimate tensile stress and Young’s modulus
CHAPTER 4: RESULTS AND DISCUSSION

4.1 Microneedle Design

Although all of the needles purchased for this experiment were forged from stainless steel, the strength of the needles varied extensively between the different arrays. The AdminPen did not physically contain solid needles, but instead there were 600um spikes adjacent to holes in the array to mimic needles. The spikes were intended to create a hole in which fluid could flow into when the plunger in the syringe was pushed.

Figure 34: “Microneedle” arrangement on circular hub. (a) Hub is composed of a sticky, plastic material and filled with foam to maintain shape (AdminMed, Adminmed.com) (b,d,c) Images taken on dissection microscope at varying magnifications to show needle and bore shape
This array was purchased for testing because it is inexpensive to fabricate and easily customizable; therefore, it could be a wise option to use instead of actual needles. However, the AdminPen immediately failed in preliminary testing. The needle hub was poorly hand-made and contained gaps around the hub that allowed the fluid to escape. The needles were comparable to the physical characteristics of aluminum foil and immediately deformed when pressed against a tissue.

Three MicronJet hubs were received in sterile packing from NanoPass. The arrays tightly screwed into the luer lock of a syringe and efficiently channeled fluid into the needles. However, all three of the NanoPass arrays deformed within the first five injections. In the first two cases, the needles immediately bent and could not be repaired without causing further damage to the device. The MicronJet microneedles were made of silicon and this may have caused the failure of the needles. Images of the damaged needles are shown below in Figure 35.
The device clearly states that it is single-use only, but in the third case, the needle broke immediately with the first injection. This device does not withstand the physical forces necessary for tissue recellularization with repeated injection.

The Micropoint Technologies 9-microneedle array was used in all preliminary and final studies, totaling about 1500 insertions. The needle was sterilized in the autoclave four times. Images were taken of the needle after these procedures (Figure 36).
After 1500 insertions, the needles no longer have a 45° beveled tip, yet all needles retain an open channel and injection ability. Use of the needle after the tip is dulled may have a negative effect on the mechanical properties of the scaffold. The needles must pierce the tissue or else the tissue will only deform against the needles and not create a channel for injection.

4.2 Experimentation: Injection Capability

The microneedle array was successful at delivering cells to the aortic cusps, but faltered when used on the decellularized carotid arteries. This leads us to believe that the density of the tissue plays a significant role on the injectibility of the tissue. With respect to the arteries, some cells were able to penetrate the elastic wall, but the majority of the cells remained on the periphery of the vessel. However, microneedles effectively distributed cells throughout the thickness and length of the cusp. Cusps are more porous
and less dense than arteries and this allowed the needles to expel cells into the scaffolds with less resistance.

Although cellular retention after injection into arteries was poor, the microneedle method still holds promise. The microneedles met all the requirements of bulk seeding except one: safety, preservation of cellular viability, reduced seeding time, minimal damage to scaffold, and repeatability were each established; however, a high seeding density was not initially observed.

These results could be partially attributed to the method used to evaluate cell density. Histology was the sole method used to determine if the scaffolds were sufficiently seeded. Histological slides are created from 5μm sections of a tissue and only expose the cells in that section. If a section was cut adjacent to where the cells reside, we may mistakenly assume that there are no cells in the scaffold. Another method to count cells, such as flow cytometry, must be used in the future to determine how many cells remain on the scaffold post-seeding.

Preliminary studies with the microneedles and decellularized arteries and cusps showed that a single 10μl bolus injection into the scaffold did not generate enough pressure to drive cells into the interior. To counter this, several 10μL injections were made in the same place to increase pressure within the scaffold. This multiple-injection method was used throughout the studies in this thesis and provided much better results. By further optimizing the injection flow rate, more cells could be injected into the scaffolds.
The needles did not exceed the depth of the tissue, and thereby eliminated the issues associated with injection by hypodermic needles. No needle holes were made that permeabilized the tissue. Figure 37 shows the tissues after they were injected with the cell suspension. Although faint, there is evidence of the 9-needle grid shown on both the artery and the cusp. The small pink dots in the tissue represent the cell suspension beneath the outer layer of the tissue. When these dots were visualized, the injection was considered to be successful.

Figure 37: Tissues post injection. (a) The black circle surrounds the imprint of the nine needles after a 20uL suspension was injected into the carotid artery scaffold (b) Manner of injection with the repeat dispenser (C) The black circle surrounds the imprint of the nine needles after a 20uL suspension was injected into the aortic cusp scaffold

Several studies have been published on the fluid dynamics of liquid flow through microneedles (Amin, 2013; Wang, 2005; Maranto, 2006). These studies have found that single microneedles inserted into skin without retraction had flow rates of 15–96 µl/h, but partial retraction of microneedles increased flow rates up to 11.6-fold (Maranto, 2006). Also, by increasing the pressure at which the fluid was injected, the flow rate could be further increased. The studies also concluded that dense dermal tissue compresses during
microneedle insertion and prevents fluid from leaving the needle tip. Due to these results, we tried to retract the needle slightly after injection. It is uncertain if this may have caused some of the cells to escape back out of the pore onto the exterior of the scaffold.

Microneedles have the potential to become the standard of cell seeding, but some adjustments must be made to improve this seeding method. The flow rate and pressure of the cell suspension leaving the syringe must be increased in order for cells to be firmly deposited into the scaffold. To accomplish this, a pressurized pump could be attached to the syringe. The pump could be set to different pressure settings to meet the requirements of various tissues; for example, a cusp would require a lesser pressure than an artery. Also, more concentrated cell suspensions could be injected into the tissue to increase the probability of cell adhesion. A larger volume deposited into the scaffold could also assist in increasing injection pressure.

4.3 Experimentation: Mechanical damage

Results of the suture retention strength test, burst pressure test, and uniaxial tensile tests showed that repeated injection with microneedles did not have a negative effect on the scaffolds’ mechanical properties. Diametrical compliance decreased with the decellularization of the tissues but did not suffer due to repeat injections with microneedles. Our lab has previously shown that tissue fixation with PGG tends to improve the strength and durability of the scaffolds, causing an increase in burst pressures and uniaxial mechanical properties.
CHAPTER 5: CONCLUSIONS

1. Microneedles can inject a high cell density in porous cardiovascular tissues, but falter when used in dense tissues.

2. Repeated injections with microneedles have negligible effects on the mechanical properties of the tissues.

3. Microneedle design, including needle height, array size, needle diameter, material, can be tailored to meet the needs of the specific tissue.
CHAPTER 6: RECOMMENDATIONS

1. The pressure at which the cells are injected must be increased in order for microneedles to penetrate dense tissues and inject cells at a clinically relevant rate. The effects of higher pressure on cell viability must then be investigated.

2. Other decellularized tissues should be investigated, such as decellularized pericardium, decellularized aortic root, and decellularized myocardium.

3. Higher seeding densities must be tested.

4. Test microneedles with openings at the side of the needle instead of the tip to decrease the chance of tissue clogging.

5. A “robot” to automate the seeding of scaffolds should be investigated. This robot should monitor seeding pressure, seeding depth, and seeding pattern across the scaffold.

6. Fresh tissue from the same batch of tissues that were decellularized should be used for mechanical testing instead of values from literature.

7. A long-term bioreactor study should be done to determine if cells proliferate, migrate, or differentiate over time. The scaffolds from each long-term time point should undergo mechanical testing.

8. Live/Dead assays should be conducted at different time points during a long-term study to determine changes in cell viability over time.
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